Genotypic and phenotypic changes in the colonic mucosa of patients suffering from longstanding ulcerative colitis

By

Mariann Friis-Ottessen

2014

Gastro-intestinal and skin tumour biology
Section for carcinogenesis

Department of Pathology
Division of Diagnostics and Intervention
Institute of Clinical Medicine
Faculty of Medicine
University of Oslo

Financial support provided by Helse Sør-Øst and Stiftelsen UNI

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 1897

ISBN 978-82-8264-927-8

Cover: Hanne Baadsgaard Utigard.
Printed in Norway: AIT Oslo AS.

Produced in co-operation with Akademika Publishing.
The thesis is produced by Akademika Publishing merely in connection with the thesis defence. Kindly direct all inquiries regarding the thesis to the copyright holder or the unit which grants the doctorate.

Table of Contents

Table of contents

Acknowledgments

The work presented in this thesis was carried out at the section for Carcinogenesis of the Gastro-intestinal and Skin tumour biology group, at the Division for Diagnostics and Intervention: Department of Pathology at Oslo University Hospital, Rikshospitalet, 2008-2014. The project was funded by Helse Sør-Øst and Stiftelsen UNI, for which I am most grateful.

I would like to express my sincere gratitude to my supervisors, Ole Petter Clausen and Paula De Angelis. Thank you for giving me the opportunity to work in your research group, and for sharing your exceptional knowledge and expertise with me. Your continuous enthusiasm, humour, and expert advice have made my work exiting, challenging, and interesting.

Next I would like to thank Aasa Rambæk Schjølberg, for technical and moral support through all of my laboratory projects, and for being a valued co-worker as well as trusting me to work independently. I would also like to thank my co-supervisor Espen Burum-Auensen for his optimism and support, and my former colleague Birgitte Lid Adamsen for her friendship and enthusiasm.

Furthermore, I am thankful to Laila Bendix, for teaching me the Universal-STELA, and for answering all my questions concerning this procedure, no matter their magnitude. I also gratefully acknowledge the contributions of my co-authors Solveig Norheim-Andersen, Steen Kølvraa, and Per Olav Ekstrøm, and Are Hugo Pripp for help in the field of biostatistics.

Acknowledgments

I am also very grateful to all my past and present colleagues here at the institute, for contributing to a friendly and inspiring work environment, and to all my friends for continuously cheering me on. Especially I am thankful to all my lunch room and coffee companions from within and outside the institute. You made a difference.

I sincerely thank my family for their tireless support and encouragement throughout my studies, and for knowing when to ask and when not to ask. And to Linda, thank you for your support, and for including me in your life in the outside world. And finally, I would like to thank Kjetil, for his endless support and encouragement during the final stages of my work. Thank you. You are the best and I love you.

Thank you for making my thesis possible.

Oslo, July 2014

Mariann Friis-Ottessen

Abbreviations

APC/C	Anaphase promoting complex/Cyclin
ATM	Ataxia-telangiectasia mutated
BFB	Breakage/fusion/Bridge
bp	Base pair
CDCE	Constant denaturant capillary electrophoresis
CIN	Chromosomal instability
CRC	Colorectal cancer
CTCE	Cycling temperature capillary electrophoresis
CV	Coefficient of variation
DIG	Digoxigenin
D-loop	Displacement loop
DSB	Double strand break
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell-sorting
FFPE	Formalin-fixed paraffin-embedded
FISH	Fluorescence in situ hybridisation
HDF	Human diploid fibroblast
hTERT	Human telomerase reverse transcriptase
hTR	Human telomerase RNA component
IBD	Inflammatory bowel disease
IGFBP7	Insulin-like growth factor-binding protein 7
IHC	Immunohistochemistry
kb	Kilo base
kDa	Kilo Dalton
LMM	Linear mixed model
LOH	Loss of heterozygosity
OIS	Oncogene induced senescence
PCI	Phenol chloroform isoamyl alcohol

PCR	Polymerase chain reaction
pg	Pico gram
PI	Propidium iodide
Q-FISH	Quantitative FISH
qPCR	Quantitative PCR
ROS	Reactive oxygen species
SAC	Spindle assembly checkpoint
SA-β-Gal	Senescence-associated β-Galactosidase
SCG	Single copy gene
SSB	Single strand break
STELA	Single telomere length analysis
T	Telomere
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
T-loop	Telomeric loop
TMA	Tissue micro array
TRF	Telomeric restriction fragment
UC	Ulcerative colitis
U-STELA	Universal-STELA
UV	Ultra violet

Papers included:

Paper I: **Telomere shortening correlates to dysplasia but not to DNA aneuploidy in longstanding ulcerative colitis**

Friis-Ottessen M., Bendix L., Kølvraa S., Norheim-Andersen S., De Angelis P. M., Clausen O. P.

BMC Gastroenterology 2014, **14**:8 (9 January 2014)

Paper II: ***TP53*/p53 alterations and Aurora A expression in progressor and nonprogressor colectomies from patients with longstanding ulcerative colitis.**

Friis-Ottessen M., Burum-Auensen E., Schjølberg A. R., Ekstrøm P.O., Norheim-Andersen S., Clausen O.P., De Angelis P. M.

Submitted Int J Mol Med.

Paper III: **Reduced hTERT protein levels are associated with DNA aneuploidy in the colonic mucosa of patients suffering from longstanding ulcerative colitis**

Friis-Ottessen M., De Angelis P. M., Schjølberg A. R., Norheim-Andersen S., Clausen O. P.

Int J Mol Med. Jun 2014; 33(6): 1477–1483.

Introduction

Ulcerative colitis

Ulcerative colitis (UC) is a chronic, inflammatory bowel disease (IBD) affecting the mucosa of the large intestine, starting from the rectum and sometimes extending throughout. Diagnosis is made by colonoscopy and histological examinations of mucosal biopsies. Progression often presents as a gradual onset, with periods of remission and spontaneous relapses with symptoms including abdominal pain, weight loss and bloody diarrhoea [1]. Symptoms of UC usually present in the teenage years or in young adults, and the disease prevalence does not differ considerably between male and female. A genetic factor to the disease has been suggested through studies of twins [2].

The prevalence of UC worldwide seems to follow a latitudinal gradient, tied to colder climate and especially chillier summer temperatures [3]. The highest incidence is found in North America and in the northern parts of Europe, but incidence rates in parts of Asia and Latin America are on the rise [4, 5]. The rising incidence rates might be an indication that environmental factors and lifestyle affect the prevalence of UC. Current treatment is often a combination of anti-inflammatory medications and dietary changes. All inflammation is treated, and the severity of the symptoms is the deciding factor for which treatment is initiated. Treatment of inflammation leads to remission in approximately 60% of patients, and a high proportion of the patients manages also to maintain this remission state [6]. If the patient develops dysplastic

areas of the colonic mucosa or has persistent, unmanageable symptoms, surgery will be necessary. It is estimated that within ten years from diagnosis one of four patients will be colectomized. Full removal of the colon and rectum is the only permanent cure for UC at present. However, even a colectomy will not guarantee a life with no more gastric trouble. Pelvic sepsis, pouch failure, and faecal incontinence are reported as complications post colectomy in about 10% of colectomy patients in a follow up examination after three years [7].

UC can be classified according to severity;

a) Ulcerative proctitis where only the rectal part of the colon is affected
b) Left-sided colitis when the inflammation expands from the rectum through the sigmoid part of the colon and to the left part of the descending colon
c) Pancolitis which includes inflammation all through the colon.

An illustration of the extent of colonic mucosa affected in the different classifications of UC is presented in figure 1.

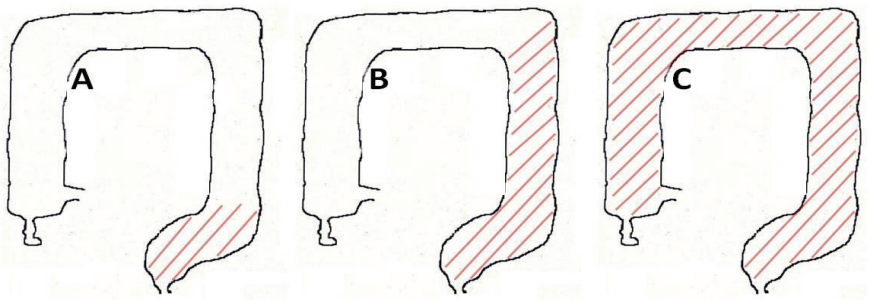


Figure 1: *The extent of colonic mucosal involvement in A) proctitis, B) left-sided colitis, and C) pancolitis.*

Fulminant colitis is a severe version of UC; this is a life threatening condition where an overpowering bacterial infection can cause toxic megacolon or colonic rupture [1, 8]. Still, the utmost consequence of UC is that it significantly increases the lifetime risk of developing colorectal cancer (CRC). This risk increases with the severity of illness and disease duration. During the first decade of disease duration no increased cancer risk is present, however, after this the risk is estimated to increase at a rate of ~1% per year. Furthermore, the risk factor is affected by the extent and severity of colitis. [9-11]. No elevated risk is detected in UC patients where only the rectal part of the colon is affected [10].

Due to the increased risk of developing colorectal cancer in longstanding UC, patients are recommended to have an increased number of colonoscopic examinations with increasing disease duration, and also if dysplastic changes as a risk factor are detected [8, 11-13]. Colonoscopy is not a perfect screening method for cancer prevention in UC, and has shown a rather poor success rate of identifying cancer at an early stage. It is also a highly invasive and expensive procedure, and it might not detect areas of flat dysplasia [14] . Lately the use of chromoendoscopy, where the dyes indigo carmine and methylene blue are applied to the colonic mucosa, has improved the value of endoscopy and has been shown to improve the detection of flat dysplasia. The applied dyes will highlight active inflammation and dysplasia. This also allows for fewer, more targeted biopsies [13].

UC is an idiopathic disease, and malignancies in a UC colon are suggested to develop in a multistep progression through inflammation, regeneration and dysplasia leading to adenocarcinoma [15]. The process also includes a number of important molecular changes such as chromosomal instability (CIN) and aneuploidy [8, 16], but although the underlying molecular pathogenesis is the subject of massive research, it is still not fully understood. The disease is also associated with an abnormal reaction to the patients' own gut microbiota, as well as environmental influences [10]. A schematic overview of malignant development in a UC-colon is shown in figure 2.

Disturbances of both the innate and the adaptive immune system contribute to dysplastic progression in a UC colon. Simply, the innate immune system might initially overreact, creating an inappropriate reaction to the gut's own microbiota resulting in inflammation of the colonic mucosa, where the adaptive immune system maintains the inflammation, rendering it chronic [10, 17].

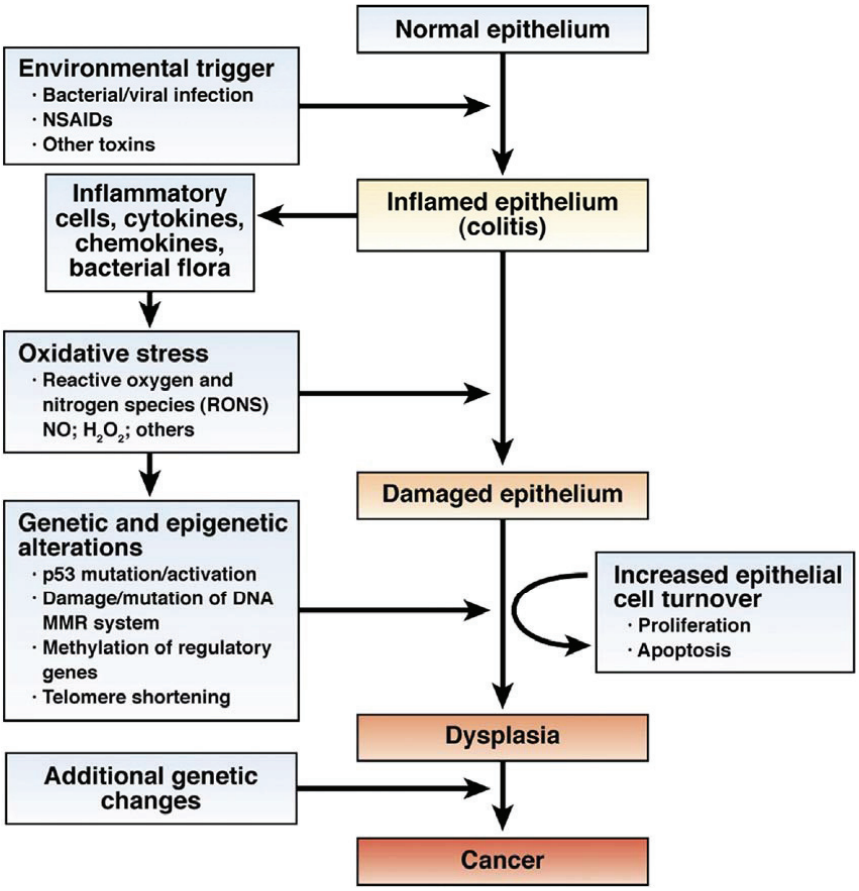


Figure 2: *A possible sequence of events from inflammation to cancer in patients suffering from ulcerative colitis. An environmental trigger (such as a bacterial or viral infection) may cause inflammation which will generate oxidative stress in the colonic environment. This may then cause genetic changes and dysplasia, and as an utmost consequence: cancer. Reprinted from Gastroenterology, volume 140, issue 6, Thomas A. Ullman, Steven H. Itzkowitz, "Intestinal inflammation and cancer", pages 1807-16, copyright © 2011, with permission from Elsevier. Figure legend is modified.*

Although all patients suffering from UC are repeatedly exposed to inflammation- derived stress in their colonic mucosal environment, only about 10% will progress to present severe pathological changes [9, 15]. These patients may be termed progressors, and typically include patients who present at least one area of low grade dysplastic development throughout the colon [11, 18]. Since the presence of DNA aneuploidy in UC can be viewed as a predisposing agent to malignancies [19], it can be argued that this is also a factor that may be used to identify progressors.

Intestinal inflammation and oxidative stress

Inflammation is defined as a biological response to an injury or a pathogen. It involves a series of complex defence mechanisms, and is divided into two main, specialized branches, namely the innate and the specialized, adaptive immune system. The initial response to an injury or a damaging agent is activation of the unspecific innate immune system, recruiting amongst other agents; leucocytes and macrophages. This activation will further recruit the specialized, adaptive immune system of T-cells and B-cells. In a healthy colon both the innate and the adaptive immune system partake in a dynamic crosstalk with the intestinal epithelial cells, to ensure maintenance of intestinal homeostasis. This dynamic crosstalk ensures protection against pathogens and allows for passage of nutrients [20-23].

In UC, the homeostatic balance in the intestine is compromised. It has been shown that both the innate and the adaptive immune systems are affected, and proceed to drive inflammation instead of fighting it [21, 22]. The chronic inflammation occurring in UC further distorts the mucosal morphology through regeneration, and may then induce dysplasia and adenocarcinomas [10, 24]. It is suggested that chronic inflammation is more likely the underlying cause of UC-related cancer risk than is any clear-cut genetic predisposition, as adenocarcinomas can develop from polypoid, flat, localized or multifocal dysplasia in an UC-affected colon [10].

Leucocytes and macrophages recruited during the initial inflammatory response produce a range of reactive oxygen species (ROS) [23, 25-27]. When the levels of ROS exceed the limits that can be processed by the endogenous antioxidant defence mechanisms, oxidative stress develops [25, 28, 29]. To further complicate the situation, inflammation may also impair the antioxidant defence mechanisms in UC colonic mucosa [28, 30-32]. It has been observed that the environment in a UC-affected colon harbours elevated levels of oxidative stress [25, 31]. Oxidative stress can lead to severe damage of the DNA by inducing both single and double strand breaks (SSB and DSB, respectively) in chromosomes. Whereas SSBs may be repaired through base excision mechanisms, a DSB can lead to apoptosis or CIN if not properly repaired through either homologous recombination or non-homologous end joining mechanisms [33]. A schematic view of the implications of oxidative stress to the colon environment of a UC-colon is shown in figure 3.

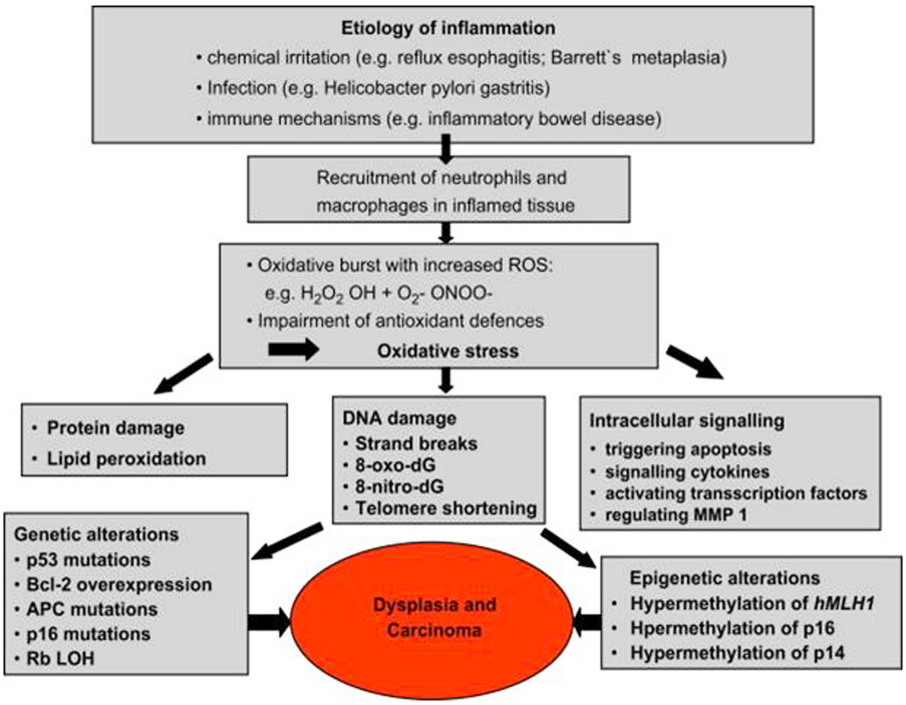


Figure 3: *Inflammation cause increased levels of oxidative stress, which may drive the development of dysplasia and cancer through DNA damage and genetic alterations. Reprinted from Pathology- Research and Practice, Volume 204, Issue 7, A. Roessner, D. Kuester, P. Malfertheiner, R. Schneider-Stock, "Oxidative stress in ulcerative colitis-associated carcinogenesis", pages 511-25, Copyright © 2008, with permission from Elsevier. Figure legend is modified.*

Damage to the chromosomes by excess oxygen might be repaired by the complex DNA damage response pathways, or if the damage is too severe; the cell might be targeted for apoptotic destruction. To allow the cells' repair mechanisms time to repair the damage induced by the excess oxygen, cell cycle checkpoints are activated. However, it has been shown that elevated levels of oxidative stress can cause a cell to bypass cell cycle checkpoints, with the result of inducing CIN and DNA aneuploidy in a cell population [33-36]. If the oxidative stress-induced break occurs in a telomeric region it is less likely to be repaired, due to a telomere-specific repair deficiency [37] and aneuploidy may develop through pathways induced by telomere biology.

Aneuploidy

Aneuploidy is defined as the karyotype of a cell with abnormal DNA content, whereas CIN is the rate of chromosomal gain or loss [38, 39]. It is acknowledged that CIN may lead to aneuploidy [35], however, not all aneuploid cells exhibit chromosomal instability during mitosis [38, 39]. Aneuploidy can further refer to either structural errors or copy-number errors in chromosomes, and an aneuploid cell will usually contain a combination of these. The leading hypothesis regarding causes of aneuploidy has been that structural errors are most likely products of chromosomal breakage [40-42], while copy number errors are achieved mainly through errors in chromosomal segregation [43, 44]. Lately, it has also been suggested that mitotic segregation errors can cause both types of aneuploidy [45].

Aneuploidy is detrimental to cell survival in non-neoplastic cells, as it will result in genetic imbalance [38]. However, nearly all types of solid tumours may display aneuploidy [46]. It is suggested that an organized and selective aneuploidisation can be present in malignant development, through the deactivation of tumour suppressor genes and activation of oncogenes, ensuring growth advantages for the aneuploid cells [38].

Aneuploidy in UC

In UC, aneuploidy is regarded an early event [19, 47-49], and may be present in both dysplastic as well as non-dysplastic colonic mucosa [19, 48]. It has been suggested to be an independent risk factor for the development of adenocarcinoma in UC [50, 51] and more than half of the colorectal adenocarcinomas developing from UC present DNA-aneuploidy [51, 52]. Aneuploidy is considered to be a major contributor to the neoplastic phenotype [53, 54].

Carcinogenesis in UC shares several features with the development of sporadic colon cancers, but the sequence of alterations differs [10]. Non-malignant mucosa adjacent to a cancer derived from a UC colon has demonstrated a higher frequency of aneuploidy than seen in colons affected by sporadic colon cancer, a feature that implies a DNA field effect presence throughout a UC colon [55, 56]. In UC mucosa aneuploid areas are often more widespread than dysplastic areas and aneuploidy is suggested to spread by clonal expansion of aneuploid subclones harbouring increasing levels of CIN, at the expense of diploid epithelial cells [57]. The frequency of UC-patients presenting aneuploidy increases with duration of the disease [19, 58].

Telomeres

Telomeres are specialized and repetitive DNA structures, located at the end of linear chromosomes, where they function as a stabilizing agent for the chromosome and as a protective agent against chromosomal ends being recognized as double strand breaks (DSB) by the DNA repair mechanisms of the cell. The human telomeric sequence consists of a repeated 6 base pair (bp) sequence; TTAGGG [59, 60], measuring approximately 8-12 kilo base pairs (kb) in newly synthesized somatic cells, when measured in newborns [61]. The telomere ends in a single stranded, 30-600 bp long G-rich overhang [60].

Chromosomal integrity and protection against the ends being recognized as DSBs are achieved by having the telomeric end form a protective loop-structure: the telomeric loop or T-loop [62, 63]. The telomeres are protected by a group of specialized proteins; the shelterin complex. Shelterin consist of six subunits (TRF1, TRF2, TIN1, TIN2, Rap1, TPP1 and POT1), and these proteins are specific in binding to either the double strand or the G-rich single strand part of the telomere [41, 64, 65]. It is suggested that the shelterin complex is active in forming, shaping and stabilizing the T-loop structure, where the G-rich single strand overhang is incorporated in the loop in a displacement loop, the D-loop, a feature important in forming the T-loop [41, 64, 65]. Figure 4 shows the structure of the telomere with a single strand overhang and the T-loop.

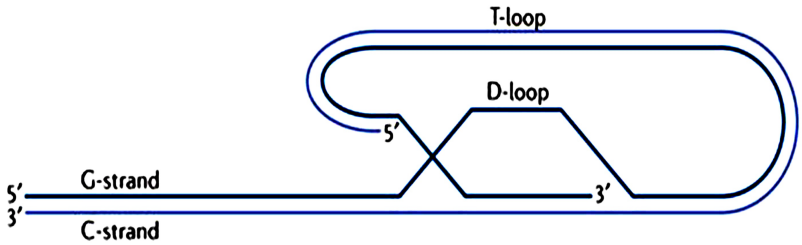


Figure 4: *The telomeric end is protected from being recognised as a double strand break by forming a T-loop, where the single strand overhang is tucked into the loop in a smaller D-loop. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology (Finding the end: recruitment of telomerase to telomeres), Copyright © 2013. Figure legend is modified.*

Due to the end replication problem the telomeric sequence is shortened with every round of cell division. This was first noticed by Hayflick and Moorhead, while working with cultured human diploid fibroblasts (HDF). They found that past 60-80 population doublings the HDFs stopped proliferating, and became large and flattened [66]. The limitation point for continuous growth under cultured conditions for HDFs was termed the Hayflick limit or replicative senescence. A senescent cell is still in a metabolically functioning state, but is no longer proliferative. Shortened telomeres have been discovered to be largely responsible for inducing replicative senescence in a cell population [67].

In absence of external influences, the cells' telomeres would only shorten as a result of the end replication problem, and at a certain limit tumour suppressors such as p53 would induce and maintain replicative senescence. However, senescence can be induced by various methods, not all involving telomere length erosion [67]. Initially it was believed that once a cell had become senescent, it could not re-enter the proliferative population. This has since been shown not to be the case. Inactivation of functional cell cycle checkpoints allows cells to bypass the point of replicative senescence [68, 69] thus allowing for further proliferation of cells with uncapped chromosomes, where the telomeres are too short to form the T-loop structure [70].

Gradual telomere erosion, cellular senescence, and crisis

Telomeres shorten via two major mechanisms. One is the gradual erosion of the full telomere body through proliferation, resulting in cellular aging and tissue maintenance. This system is dependent on functioning cell cycle checkpoints. If these checkpoints are defective for any reason, cells can continue to proliferate past the Hayflick limit towards crisis, further shortening the telomeres to a point where they become critically short and fail to assemble the T-loop [70-72]. Figure 5 illustrates the effect of gradual erosion of telomeres.

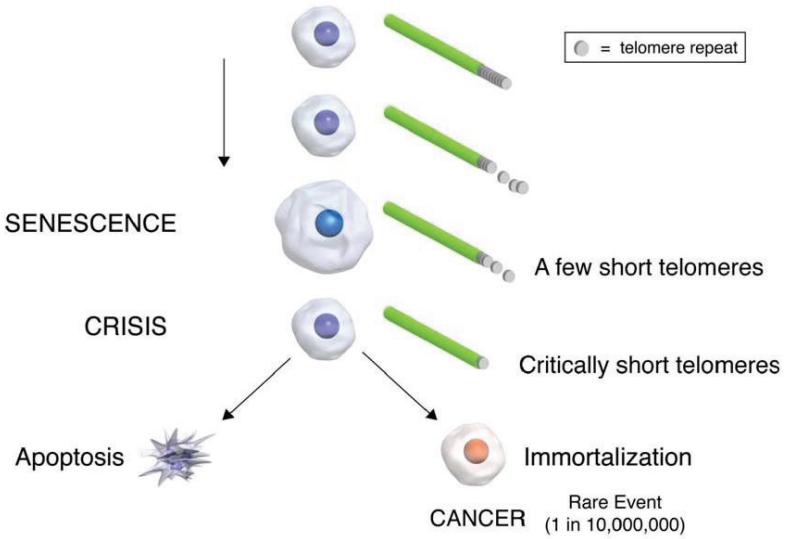


Figure 5: *The telomeres of proliferating cells shorten by each round of cell division. With functional cellular checkpoints, telomere shortening initiates aging: senescence. Without functional checkpoints the cell might progress until crisis, with increased rates of CIN and aneuploidy. Crisis ends in massive apoptosis, or in rare events: further progression towards cancer. Reprinted from FEBS Letters 2010, Volume 584, issue 17, Jerry W Shay and Woodring E Wright, "Telomeres and telomerase in normal and cancer stem cells", pages 3819-25. DOI: 10.1016/j.febslet.2010.05.026. Figure legend is modified.*

Crisis produces increased amounts of aneuploidy and massive cell death through apoptosis. In very rare events, a limited amount of cells, estimated to be one in ten million, cells are observed to survive crisis by activation of a telomere maintenance mechanism, thus achieving cellular immortality [70-72].

Sporadic telomere shortening and breakage/fusion/bridging

Sporadic telomeric shortening is the other mechanism for achieving shorter telomeres. This mechanism differs from gradual erosion by striking randomly and by the ability to leave either one or a few chromosomes at a loss for telomeric capping, regardless of cellular age and proliferative stage. The presence of telomeric deletions that could not be accounted for by telomeric erosion and cell proliferation was first indicated in a cell line harbouring a tagged chromosome end [73]. It was further suggested that it might not be shortened mean telomere length that induces senescence, but rather one or a few critically shortened telomeres, and that these could not be accounted for by the gradual erosion of telomeres [74-76].

Sporadic uncapping of a chromosome by the loss of a telomere may cause fusion of sister-chromatids during chromosome amplification [41, 75, 77, 78]. As the spindle apparatus pulls at the fused chromatids they will break, and usually at a different location than the fused ends. The resulting aneuploid daughter cells then have chromosomes with inverted amplifications or permanent deletions. Both daughter cells still harbour chromosomes with broken ends, capable of fusing with other broken chromosome ends, and a breakage/fusion/bridge (BFB) cycle will be established. This cycle will accumulate chromosomal instability and may thus create aneuploid populations [40, 77]. An illustration of telomeric induced BFB is shown in figure 6. The induced BFB-cycles will stop only if a new telomere is achieved, either through activation of a telomeric lengthening mechanism, or by telomeric acquisition as a result of translocation [77, 78].

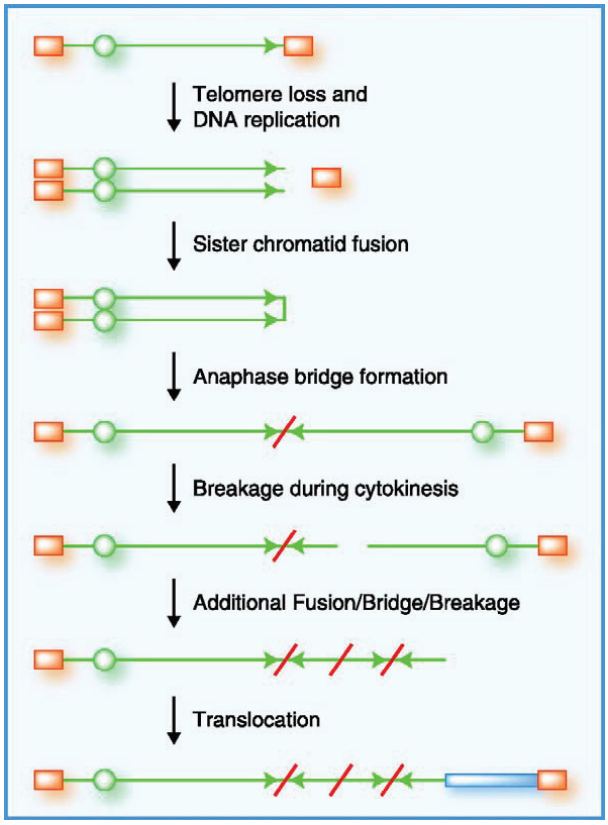


Figure 6: *Chromosomal instability through breakage/fusion/bridge cycles. An abrupt shortening of telomeres causing uncapped chromosomes, may induce sister chromatid fusion. The fused chromatids will form a bridge during anaphase, and a chromosome break follows. The daughter cells will thus inherit chromosomes with broken ends, capable of fusing with other broken chromosome ends, and a BFB cycle will be established. This cycle accumulate chromosomal instability and may create aneuploid populations. Reprinted from Cancer Research, Copyright © 2010, Volume 70, Issue 11, pages 4355-59, John P Murnane, "Telomere loss as a mechanism for chromosome instability in human cancer", with permission from AACR. Figure legend is modified.*

BFB cycles may also involve fusion of two separate chromosomes. This is rarer than sister-chromatid fusion, estimated at about one chromosomal fusion BFB for every fifth or even tenth sister-chromatid fusion. Chromosomal fusion may happen at any time during the cell cycle, as it is induced by either the loss of two telomere caps at the same time, or from sporadic double strand breaks. Also, the resulting genotype resulting from chromosomal fusion BFB is more varied, as it is dependent on both the site of the initial DSBs or two separate chromosomes independently uncapped, as well as each cycle will involve two centromeres and a double anaphase bridge [41, 75, 77].

Sporadic telomere shortening is attributed to several mechanisms [79], and oxidative stress is one of the mechanisms that has received much attention in the quest to unravel these mechanisms.

As previously mentioned, oxidative stress is known to be a product of active inflammation, reported in UC-colonic mucosa [25] and may act upon the chromosome by generating both SSB and DSB [33, 37]. If this chromosomal break occurs either in a telomere or in the sub-telomeric region of a chromosome, the break is less likely to be repaired [37, 80, 81], and may thus lead to sporadic telomeric loss. Oxidative stress has also been shown to make single strand breaks in a telomere, at the G-rich site. This SSB may then induce a severe shortening of the telomeric sequence during the next round of DNA replication [37, 82-84].

Telomere shortening in UC

In a UC-affected colon the mucosal lining is exposed to extreme conditions not seen in the mucosal lining of a healthy colon. It has been demonstrated that it is subjected to both enhanced cellular proliferation [85, 86], a situation capable of not only shortening mean telomere length, but also of elevating the possibility of mitotic slippage, and to oxidative stress [25, 31]. Thus, the colonic mucosal cells of a UC patient hold every chance of telomeric-induced CIN and aneuploidy. It has also been demonstrated that shortening of mean telomere length in a UC colon is accelerated compared to non-UC affected colons, and occurs mainly during the first decade following UC onset [87, 88]. Telomere shortening occurs mainly in the epithelial cells [89], and has also shown links to development of aneuploidy and CIN [49, 90].

Telomere shortening and cancer development

Telomeres have been widely studied in association to cancer development due to the induction of CIN and aneuploidy. Both CIN and aneuploidy are frequently present in solid cancers. However, it is also known that telomeric shortening and DNA-damage induce either cell senescence, or if the DNA damage is irreparable; apoptosis and cell death. The switch between the two outcomes has been shown to reside in the cell cycle checkpoint machinery [40, 91]. Specifically the mitotic spindle checkpoint is the mechanism to block anaphase if the chromosomes are not correctly arranged and secured to the spindle apparatus [44, 92-94]. In cells carrying functional checkpoints, telomere shortening can be regarded as a protection against tumour development, as the affected cell will be halted and if repair is not achievable;

either eliminated by apoptosis, or banished to senescence. If the cell cycle checkpoints are for some reason impaired, the cell might continue proliferating until crisis is reached, with shortened telomeres enabling BFB-cycles and increase levels of CIN and aneuploidy. To survive this stage the cell needs to activate some sort of telomeric maintenance mechanism, usually telomerase and it may then evolve into a cancer cell [40, 95]. Indeed, the majority of human cancers show telomerase activity [96, 97]. Two pathways initiated by shortened or lost telomeres are showed in figure 7.

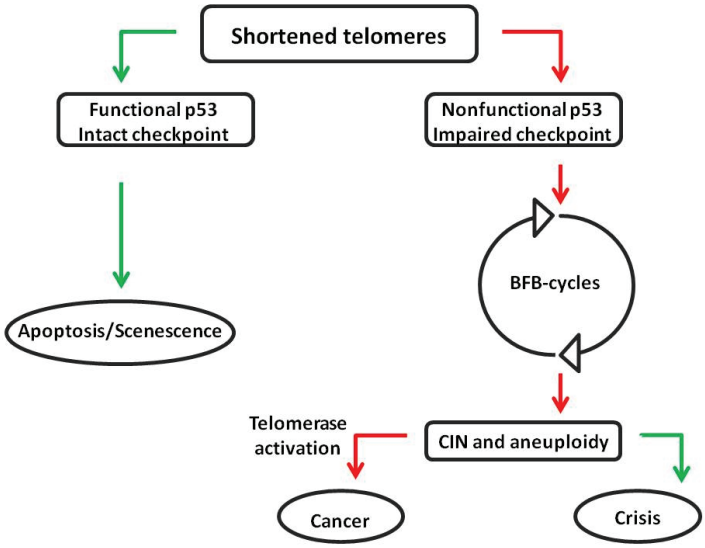


Figure 7: *The consequences of telomere shortening depends on cell cycle checkpoints and signalling pathways. The result may be either protecting the cell against malignancies (green arrows) or driving malignancies (red arrows).*

The spindle assembly checkpoint (SAC)

The spindle assembly checkpoint (SAC) is one of several cell cycle checkpoints ensuring that each phase of the cell cycle is accurately completed before commencing the next phase. It is a complex signalling cascade ensuring that chromosomes are duplicated, properly aligned at the center of the cell during metaphase, and securely attached to a spindle microtubule before entering anaphase and cytokinesis. This is crucial to ensure proper chromosomal segregation to the two resulting daughter cells. The SAC components include Mad1, Mad2, BubR1, Bub1, Bub3, and Mps1, and will block the function of the anaphase promoting complex/cyclosome (APC/C) if any irregularities are detected [44, 98-100].

During mitosis, chromosomes cannot be transcribed due to their condensed form, and any damage introduced to the DNA at this time cannot be repaired. Because of this halting the cell prior to entering anaphase may cause reduced viability. Therefore, multiple drugs have been developed to target either entry or exit from mitosis, or to induce DNA damage to the condensed chromosomes, thus underscoring the importance of understanding the function of the components of the SAC complex [101]. Deregulations of the SAC-complex via genetic alterations are connected with development of CIN and aneuploidy [43, 44, 102, 103]. An illustration of the cell cycle is presented in figure 8.

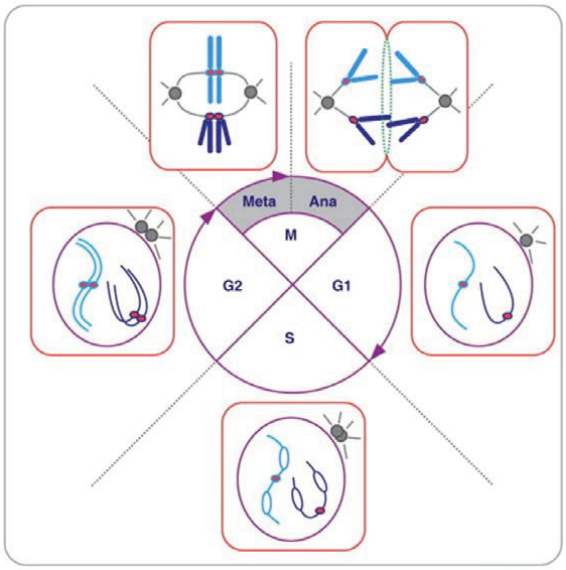


Figure 8: *The cell cycle (G1, S, G2, and M). The spindle assembly checkpoint (SAC) is activated if chromosomes are not properly aligned during mitosis (M). The chromosomal arrangement during the cell cycle is represented by two sets of chromosomes (light and dark blue), and the microtubule placement is indicated by grey circles. Checkpoints are indicated by purple arrowheads. During mitosis the chromosomes are condensed, and DNA repair is not possible. Modified from Current Biology 2012, Volume 22, issue 22, Pablo Lara-Gonzales, Frederick G Westhorpe, Stephen S Taylor, "The Spindle Assembly Checkpoint", pages R966-80. DOI: 10.1016/j.cub.2012.10.006. Figure legend is modified.*

Regulation of the SAC complex

In mammals, the SAC is assembled and activated by the presence of unattached kinetochores as the cell enters mitosis. It is then kept constantly active during mitosis, and deactivated only when all kinetochores are satisfactorily attached to a spindle microtubule, and the chromosomes are aligned along the centre of the cell. By this time, the SAC is deactivated, and the APC/C complex can continue to activate separin protease, which in turn separates the sister chromatids [43, 101, 104].

In a cell harbouring altered concentration, or mutated variants of a SAC-component, the function of the checkpoint is compromised. Both increased and decreased levels of Mad2 predispose a cell to developing aneuploidy. Overexpression of BubR1 may provide a protective mechanism against aneuploidy, whereas insufficient amounts of Bubr1 may facilitate the development of aneuploidy [105].

The SAC seems to also be influenced by oxidative stress. Elevated levels of oxidative stress in cells arrested in mitosis have been shown to induce loss of cyclin B1 stability, or inhibition of cyclin B1 entry through the nuclear membrane in cultured cells [27, 34]. Also, both Mad2 and BubR1 signalling are affected by oxidative stress in cultured fibroblasts and in gastric cancer cell lines. An accumulation of both BubR1 and Mad2 as a response to oxidative stress may contribute to CIN and aneuploidy, possibly in a p53 dependent manner [106]. Several important regulators other than the SAC components have been identified, including the tumour suppressor p53 (*TP53*) and the kinase Aurora A [107-110].

Aurora A

Aurora A is one of the three identified human cell cycle regulation serine/threonine kinases of the Aurora family. All three members (Aurora A, B and C) are frequently overexpressed in cancers [111]. Elevated expression of Aurora A is also detected in UC-colonic mucosa [112].

Aurora A is tightly associated with the SAC-signalling cascade through centrosome maturation, and regulated through phosphorylation and ubiquitination [108, 111]. Through its association with the centrosomes and spindle apparatus, Aurora A is assumed to play a role in the development of aneuploidy in malignant cells, [111, 113, 114]. Decreased levels of Aurora A may cause spindle defects leading to development of tetraploid cells which in most cases ends in apoptosis [111], whereas overexpression of Aurora A is reported to induce multiple centrosomes and bypass of the SAC, as well as failure of the G2 checkpoint and cytokinesis. In fact, Aurora A is listed as an oncogene since overexpression is linked to excess centrosomes and abnormal spindle formations, leading to a disruption of SAC function [109]. Overexpression of Aurora A is also shown to mediate impaired cellular response to chemotherapeutic agents targeting the mitotic checpoint in colorectal cancer [115]. Overexpression of Aurora A is frequently seen in cancers, including sporadic colorectal cancer [111, 113, 116], and there are also indications that expression is elevated in UC-related colon cancers and in non-cancerous lesions from UC mucosa [112]. The elevated expression detected in UC non-cancerous lesions may be a consequense of the elevated cell proliferation reported for the colonic mucosa of UC-patients [85, 86].

The gene coding for Aurora A is located on chromosomal arm 20q13.2 [113]; 20q amplification has been shown to be the most frequent chromosomal amplification in sporadic colorectal cancers [117]. An association with development of CIN in sporadic colorectal cancers has also been demonstrated [114]. However, Aurora A is probably not a genuine oncogene, as overexpression of Aurora A alone is not enough to transform primary cells, and to act as an oncogene further alterations, such as inactivation of p53 are likely necessary [109, 118, 119].

As a kinase, Aurora A phosphorylates substrates, and p53 has been identified as one such Aurora A substrate. Under normal, non-malignant circumstances, Aurora A and p53 are involved in a negative feedback loop [113]. Aurora A may phosphorylate p53 at serine residue 215, rendering it unable to bind to DNA for transcription, or in position 315 facilitating ubiquitination of p53 by Mdm2 and thus ensuring degradation of p53 through the proteasome complex [107, 120]. This might be relevant for normal cell proliferation, allowing cells to pass through G2-M cell cycle checkpoints. In addition; p53 negatively regulates Aurora A in both a transcriptional and post-translational manner, thus indicating a feedback loop regulation [108, 121]. However, it seems that only wild-type p53 has the ability to regulate Aurora A [121]

Aurora A overexpression seems insufficient for malignant development and a regulation feedback loop containing Aurora A and p53 has been identified. It is therefore speculated that this regulation is needed for a cell to proceed through the cell cycle in a normal manner, and that disruption to the feedback loop may allow a cell to progress towards a malignant state. This theory can be further supported by the multiple reports of elevated levels of Aurora A, and also loss of/inactivation of p53 in multiple cancers.

p53

p53 is a transcription factor and a tumour suppressor, coded for by the *TP53* gene, which is located on chromosomal arm 17p13.1. Under normal conditions p53 is inactive and has a very short half-life of approximately 15 minutes [122, 123], before it is subjected to poly-ubiquitination, targeting it for degradation by the proteasome [124].

The general effect of p53 induction is to limit cell growth, and p53 is probably the protein most often inactivated in human cancers, resulting in cells with abrogation of apoptosis. Due to the frequency of elevated p53 protein levels demonstrated in many cancers, and because it seems to cooperate with other, known oncogenes resulting in malignant transformations; p53 was at first characterized as an oncogene. It took almost ten years from its discovery for it to be classified as a tumour suppressor gene in 1989 [125, 126].

p53 is activated by multiple agents causing stress to the cellular environment. DNA damage and oxidative stress are both known causes of p53 activation. Once activated p53 will through regulation of a range of possible target genes, induce an appropriate response to the stress stimulant. The response pathway mediated is dependent on cell type, cellular environment and possible other oncogenic alterations. [124, 127]. Various stress-causing agents and responses mediated through p53 are presented in figure 9.

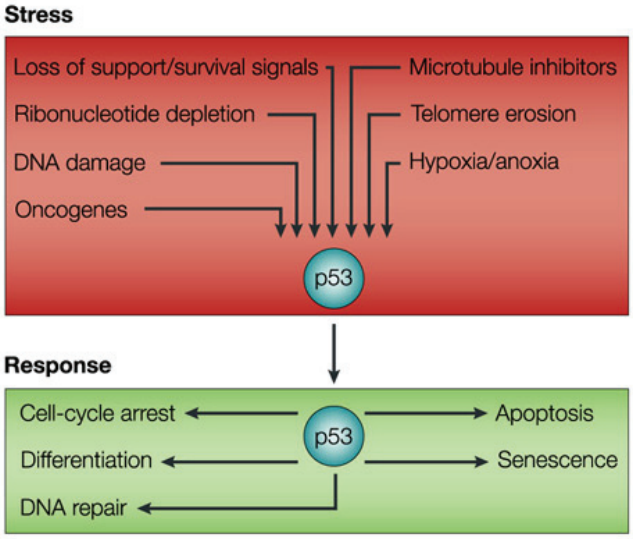


Figure 9: *Stress-causing agents and p53-induced responses. The general effect of p53 induction is to limit cell growth, and the choice of response induced by p53 will depend on cell type, environment, and other oncogenic alterations. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews, Cancer (Live or let die: the cell's response to p53) Copyright © 2002. Figure legend is modified.*

p53 levels or activity, can be influenced by multiple mechanisms, including protein regulation and mutations of the *TP53* gene. The most frequent *TP53* mutations seen in cancers are missense mutations, point mutations resulting in single amino-acid substitutions, producing non-functioning p53 proteins, often with considerably lengthened half-lives. In addition to this, loss of heterozygosity (LOH) is a common *TP53* gene alteration, causing elimination of the remaining wild-type allele [123, 127, 128]. In sporadic colorectal cancers this combination of LOH and *TP53* point mutations is a common way of abolishing p53 expression, and is considered to be a late event in tumourigenesis; occurring in the shift from dysplasia to adenocarcinoma [129].

As *TP53* mutation frequently results in proteins of longer half-life than wild-type p53, mutant p53 protein may be detected by immunohistochemistry (IHC). In sporadic CRC the p53 levels are often quite high, and do not always correlate to detected *TP53* mutations [130, 131], possibly indicating that accumulation could also be caused by wild-type p53 accumulation due to a deactivated MDM2; a known regulator of p53, or by phosphorylation pathways [124].

p53 expression strongly correlates with aneuploidy in CRC [132, 133]. It has also been shown that p53 accumulation in CRC does not correlate to common clinicopathologic variables such as age and localisation of adenocarcinoma in the colon, and it is therefore suggested that p53 inactivation is an important event in the development of adenocarcinomas in colorectal cancer, regardless of clinicopathologic features [134]. *TP53* mutations have demonstrated an association to tumour site, as a significantly elevated frequency of mutations is detected in tumours originating from the left and distal parts of a colon [135, 136]. *TP53* mutations have also been shown to associate with tumour progression in sporadic colorectal cancers and not to correlate with p53 protein accumulation [137]. The location of mutation may also introduce a possible explanation for some of the lack of correlation between mutations and accumulated protein, since some missense mutations outside exons 5-8 will not be detected by the most conventional methods of analysis. The mutations detected might also be nonsense mutations, resulting in no detectable p53 protein [135, 136, 138, 139]. It is hypothesized that in human cancers 20 % of *TP53* mutations are nonsense or stop codon mutations, resulting in lack of p53 detection by IHC [128].

In UC progression p53 alterations are considered early events, even prior to any detectable dysplasia, and possibly an initiating factor of neoplasia [10, 11, 140, 141]. Both *TP53* mutations and p53 protein expression are considered promising biomarkers for cancer progression in UC patients, as both mutations and elevated p53 levels are present in non-dysplastic mucosa adjacent to dysplastic or cancerous tissue [17, 142]. Analyses of p53 status in UC by IHC have revealed increasing levels of p53 with increasing severity of dysplasia, as well as elevated levels of p53 in biopsies collected years prior to malignant development [140, 143, 144]. p53 expression has also been shown to correlate to the presence of aneuploidy in UC [145]. Mutation analyses have shown that *TP53* mutations may be an initiator mutation in UC cancerisation, as they were detected in non-dysplastic colonic crypts surrounding dysplastic crypts [141].

p53 is a major player in the induction and maintenance of a senescent state if telomeres are shortened or even damaged, and lack of p53 expression might cause a cell to circumvent the Hayflick limit, thus allowing cells to keep dividing towards crisis [41]. Once cells hit crisis, they will die, unless some sort of telomere maintenance mechanism is activated. This telomere maintenance mechanism occurs in approximately 1 in 10 million cells, and has been identified to be telomerase in 90 % of these cases [71].

Telomerase

Telomerase is a reverse transcriptase ribonucleoprotein and a holoenzyme, composed of a large, catalytic subunit; telomerase Reverse Transcriptase (TERT), a smaller, functioning Telomerase RNA component (TR), and several accessory proteins, including dyskerin and NOP10 for the assembly, stability and function of the holoenzyme [95, 146, 147]. Telomerase extends the telomere sequence by adding telomeric repeats to the 3'end of the telomere as an enzymatic activity. The larger subunit TERT catalyses the action of adding telomere DNA repeats to the telomere end via the RNA-template carried by the TR-subunit. In most normal human somatic cells telomerase activity is repressed [93, 147, 148], ensuring telomeric shortening for the cell to reach the natural limit of replicative senescence.

The telomerase enzyme was first discovered in 1985, in the ciliate protozoan *Tetrahymena thermophila* [149]. It has since been discovered that the catalytic subunit of the enzyme, TERT, is conserved in protozoans, yeast, and mammals. Since telomerase synthesizes DNA from a RNA-template; the sequence is also shared with retroviruses [150]. Human TERT is coded for by *TERT*, located distally on chromosomal arm 5p15.33 [147] It is the assumed limiting factor for telomerase assembly, being scarcely expressed in somatic cells. The RNA-component, TR, on the other hand is ubiquitously expressed [147]. Nonetheless, it has been demonstrated that both subunits are required for enhancement of the proliferative potential of a cell [151].

Telomerase activity in non-malignant tissue is mainly detected in germ line cells. However, some somatic niches such as the bottom part of colonic crypts and the basal layer of the epidermis, have demonstrated telomerase activity [147, 152-154]. hTERT has been suggested to shorten the longest telomeres, and possibly elongate only the shortest telomeres in a cell, thus maintaining a balanced length for a cell's telomeres. To shorten the longer telomeres hTERT is most likely recruited to the telomere by the shelterin protein TPP1, and acts separately from the hTR-subunit, which is not needed to shorten the telomere [155, 156].

Telomerase in cancer and UC

Telomerase activity is reported in most human solid tumours [96, 97]. Activation of telomerase will help cells achieve replicative immortality, one of the featured hallmarks of cancer [157]. It is suggested that telomerase, just like telomeres, may also exhibit tumour suppressor roles; as telomerase activity may hinder the activation of oncogenes through synthesizing new telomeres onto uncapped chromosomes thus halting BFB-cycles and CIN-accumulation [158]. Nevertheless, telomerase activity seems first and foremost to be a contributor to the immortalisation of tumour cells, and most metastases are comprised of cells positive for telomerase activity [159].

Chromosomal arm 5p, containing the gene coding for hTERT, is frequently amplified in cancers. This amplification of the *TERT*- locus might be a contributing factor for the activation of telomerase and subsequent immortalization of a malignant cell [160]. 5p amplification is one of the more common chromosomal gains detected in sporadic colorectal cancers [117] and in aneuploid lesions from UC-colons [161]. However, it is suggested that even subtle variations in the *TERT* sequence might have major impacts in the function of TERT [160], implying that some cells may have altered hTERT functions without 5p amplifications.

Telomerase activity has been shown in colorectal cancer [162], however reports on telomerase activity in pre-malignant tissue, such as the mucosal lining of a UC colon, vary throughout available literature [86, 163-168].

Detection of malignancies in ulcerative colitis

Biomarkers

Both molecular and genetic changes in the colonic mucosa of patients with UC are the subjects of extensive research in attempts to unravel the development of dysplasia and cancer in UC, thought to arise from a combined effect of inflammation, telomere damage, genetic instability and senescence [19, 49, 87, 164]. To detect dysplasia in a UC-colon by colonoscopy, it is estimated that more than 30 biopsies are needed, as the colon presents a large surface area. As only a sub-group of approximately 10% of UC patients will develop dysplastic changes in the colonic epithelium, a large patient group is thus put through unnecessary invasive testing, as no external characteristics that can differentiate between progressors and nonprogressors exist to date.

A biomarker refers to a measurable indicator of a condition. As methods for analysing both genetic and molecular changes in tissues are constantly being developed and improved, more and more candidate biomarkers of different conditions are emerging. However, the quantification of most biomarkers also makes them difficult to establish for clinical purposes. In UC, stable and reliable biomarkers for pre-neoplasia are in demand, and several presented suggestions are indeed promising. These include senescence markers and screening for *TP53* mutations, as well as telomere shortening and the presence of CIN and aneuploidy [17, 169].

Oncogenic field effects have been identified where dysplastic changes of the colonic mucosa in one area of a UC-colon may affect the molecular environment throughout the colon. The clonal expansions of known oncogenes have been identified in non-dysplastic areas [141, 170]. Several of these oncogenes have thus been proposed as biomarkers of early neoplasia in UC. However, the identification of biomarkers in UC is challenging, as the disease is multifocal, and further complicated with excess levels of oxidative stress and inflammation.

Telomere shortening has been suggested as a potential biomarker for sporadic colorectal cancers, provided that the p53 expression is normal and the mismatch repair mechanisms are intact [171]. The presence of shortened telomeres has also been suggested as a possible biomarker for chronic inflammation [172]. The use of telomere shortening as a prognostic biomarker may prove difficult since telomere length is individual and possibly affected by multiple parameters [173-176]. Also, the result of telomere length investigations has been shown to depend on the type of DNA extraction method used [177]. In UC, it is also important to acknowledge that telomere length shortens more rapidly within the first ten years of active disease [87, 89].

Main aims of the study

- To investigate possible differences between progressor and nonprogressor colons of patients with longstanding UC, with focus on the amount of ultra short telomeres, mean telomere length, telomerase and proteins interacting with the spindle assembly checkpoint (SAC), namely Aurora A and p53

- To investigate whether the same variables could be associated with development of aneuploidy or dysplasia within progressor colons

Methodological considerations

The following section contains description and discussion of the materials and methods chosen for this study. Detailed descriptions of the methodologies used are given in the individual papers.

Ulcerative colitis patients

We examined colectomy specimens from 30 patients who had all suffered from UC from 10-30 years. Colons were resected at the Department of Surgery, Rikshospitalet, in the period 1985-1994. All patients had developed pancolitis. The patient list includes both males and females, and the patients' ages when symptoms were first presented varied from 10 to 60 years old.

Colectomies and histopathology

At least eight locations from each colectomy specimen were examined. Histological examinations were conducted by experienced pathologists (OPFC, SNA) on sections stained by haematoxylin/eosin. Each section was classified according to the prevailing mucosal morphology. The histopathological diagnoses used were non-dysplastic mucosa, mucosa indefinite for dysplasia, mucosa with mild, moderate, or severe dysplasia, and adenocarcinoma. The pathologists judged the mucosal morphology separately, and in cases where the diagnosis differed, consensus was reached by studying these cases under a multi-headed microscope.

Use of flow cytometry to measure DNA ploidy status

DNA content analysis by flow cytometry was performed on mucosal cells harvested from each location adjacent to the section fixed for the morphological examination. Fluorescence-activated cell sorting (FACS) is widely used for analysing and sorting cells from a mixture of cells in suspension. Since a flow cytometer is able to analyse thousands of cells per second using lasers to detect specific cellular phenotypes (e.g. size or granularity) in cell samples suspended in a stream of fluid, it is used routinely as a diagnostic tool and in basic and clinical research.

In the present work Vindeløv's procedure was used to stain cellular DNA. This procedure allows for the analysis of preserved, alcohol fixed cells. Nuclei are isolated using a detergent-trypsin method and are subsequently stained for their content of DNA using propidium iodide (PI). PI is a fluorescent dye that intercalates into and binds double-stranded DNA, with little to no sequence preference. It also binds to double-stranded RNA; RNAse is included in the procedure to eliminate double-stranded RNA and thus its potential contribution to fluorescence measurements. PI fluorescence emission from flow cytometrically-analyzed nuclei represents their DNA content. Fluorescence signal intensity is used to determine whether a population of cells is diploid (normal DNA content) or aneuploid (abnormal DNA content due to gain or loss of chromosomes) [178].

DNA contents of all eight lesions of the colectomy specimens were determined by flow cytometry in 1996-97 using a FACStar Plus flow cytometer equipped with a 488 nm argon ion laser (BDIS, Spectra Physics, Mountain View, CA, USA). The samples were subsequently stored in 70% EtOh at -20°C until we repeated the analyses to control for DNA quality before commencing with further analyses. This was done using a FACS Calibur laser flow cytometer (Becton Dickinson). The later ploidy analyses were in agreement with the original ones, indicating that storage of alcohol-fixed cell samples at -20°C for more than ten years did not impact on DNA quality. An example of the original DNA-analysis and the repeated analysis is presented in figure 11.

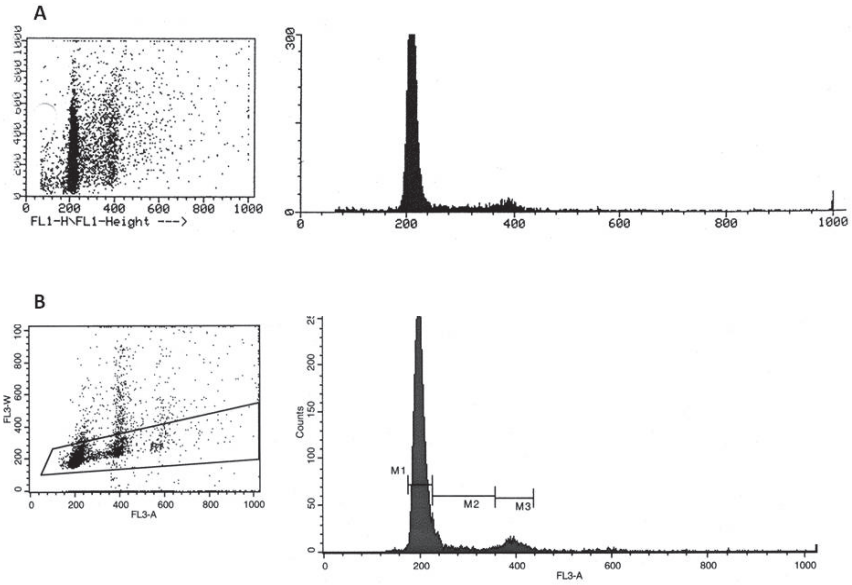


Figure 10: *DNA-content of a diploid UC-lesion as measured by flow cytometry: A) original sample, measured by a FACStar Plus cytometer, and B) repeated measure using a FACS Calibur cytometer.*

DNA isolation

DNA isolation was performed using phenol, chloroform isoamyl alcohol (PCI), a very efficient method of DNA extraction, as it produces high-quality DNA of high molecular weight. This is useful for many downstream methods. Since our analyses were focused on telomeres, we did not want excess stress to the DNA samples, so vortexing was done carefully, and a minimal amount of pipetting was done so as not to cause excess DNA-strand breakage. It has been demonstrated that PCI-based DNA isolation and high salt methods result in longer mean telomere length by qPCR analyses than does silica based spin column methods to isolate DNA [177].

Telomere length evaluation

Mean telomere length analysis

Several methods exist for the analysis of telomere length. Each method has both advantages and disadvantages, and which method to choose will depend on the material and size of the population to be studied [179-181].

Telomeric Restriction Fragment (TRF) assay is the oldest method of telomere length estimation. DNA is digested by restriction enzymes before the fragments are separated by gel electrophoresis and visualized by Southern blotting. TRF gives an estimate of telomere length [59] and might also underreport shorter telomeres in the measured sample, as not enough probe can bind to the shorter fragments [182].

Assessment of mean telomere length can also be performed using fluorescent hybridisation measured by a flow cytometer (Flow-FISH). With this method the fluorescent signal of a telomeric probe hybridized to telomeres in the sample is measured, together with measurement of cell size. The method requires calibration using cell lines with known telomere lengths [183] or by incorporating cell populations with known telomere length into the sample [184]. It is a rather efficient method of determining mean telomere length in a cell sample [184, 185]. Both methods however, require quite large amounts of DNA (TRF) or cells (Flow-FISH), and were therefore unsuitable for our rather sparse material.

qPCR is a method for estimation of the mean telomere length by measuring telomeric signal and a single copy gene signal in comparison to a reference DNA sample [186]. As this method was used to examine mean telomere length in our samples it is discussed below.

Mean telomere shortening cannot fully explain the telomere association with CIN and aneuploidy. Since excess shortening of one or more telomeres can cause both senescence and chromosomal rearrangement [74, 76], it has become clear that the frequency of critically short telomeres is also a feature to examine, along with mean telomere length.

Single telomere length analysis

Quantitative fluorescent *in situ* hybridisation (Q-FISH) on metaphase spreads can detect critically short telomeres, but needs, as the name implies, metaphase spreads [187], the production of which are quite laborious. Q-FISH on interphase cells allows for telomeric measurements of interphase cells. Telomere length has also been measured from single cells in archival tissue in paraffin sections [188-190]. Q-FISH methods are in general limited by their labour-intensive nature. High throughput Q-FISH (HT Q-FISH) makes it possible to assess the telomeres in large sample series, by culturing cells in 96-well plates, and applying the premises from interphase Q-FISH [191], but the method is thus dependent on viable, unfixed cells.

Single Telomere Length Analysis (STELA) is a PCR-based method of quantifying individual telomeres[192]. STELA makes it possible to measure the variants of telomeric length comprised of individual chromosomes present in a DNA sample. As this method is PCR-based, it is not limited by the amount of DNA, or by the availability of live cells. This method anneals a linker, the Telorette, to the G-rich 3' overhang of the telomere. This Telorette is further ligated onto the 5' end of the complimentary strand, tagging the end of the telomere. The Telorette contains a known, non-telomeric sequence, enabling it to function as a primer site for the PCR reaction. The limiting agent for STELA is the upstream primer, as it needs the subtelomeric region to be known. The subtelomeric regions of human chromosomes have proven difficult to sequence, since they contain frequently-repeated sequences. Chromosomes also seem to share some of the subtelomeric sequences between them. This makes primer-design somewhat troublesome; and this procedure can only be applied to a limited set of chromosomes.

None of the above mentioned methods of measuring the shorter telomeres can give an estimate of the general distribution of the critically short telomeres in a DNA-sample, which is what Universal STELA can do.

Universal STELA

The Universal STELA, U-STELA, is an adaptation of the Single Telomere Length Analysis (STELA) [193]. The method suggests a way to avoid the limitation of chromosome specificity induced by the internal primer used in the original STELA. The U-STELA includes an additional annealing and ligation step, where a set of specially designed oligo-sequences: the panhandle oligos, are added to the proximal end of the telomere, prior to the Telorette-oligo being ligated to the distal, single strand overhang of the telomere end. By including this additional ligation step, the method enables both ends of the telomeres to contain known sequences. Primers for both ends can then be facilitated, and the PCR will amplify all the telomeres from the sample analysed. The U-STELA workflow is outlined in figure 11.

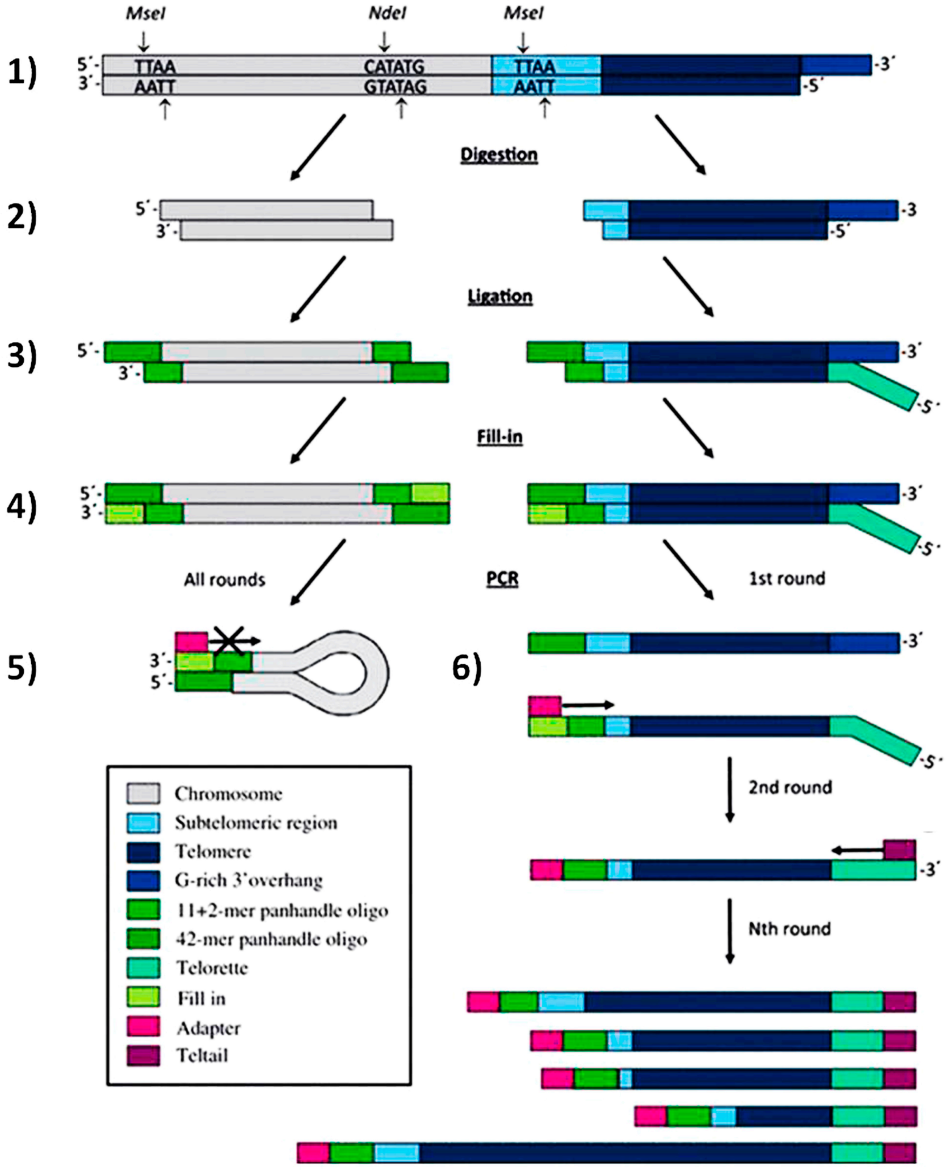

MseI
NdeI
MseI
1)
5´-TTAA
3´-AATT
CATATG
GTATAG
TTAA
AATT
-3´
-5´
Digestion
2)
5´-
3´-
-3
-5´
Ligation
3)
5´-
3´-
-3´
-5´
Fill-in
4)
5´-
3´-
-3´
-5´
All rounds
PCR
1st round
5)
3´-
5´-
6)
-3´
-5´
2nd round
-3´
Nth round
Chromosome
Subtelomeric region
Telomere
G-rich 3'overhang
11+2-mer panhandle oligo
42-mer panhandle oligo
Telorette
Fill in
Adapter
Teltail

Figure 11: *Principles of the U-STELA procedure:*

1) *Extracted DNA is digested by restriction enzymes that cut the genomic sequence, and the subtelomeric region.*

2) *The result is genomic fragments (grey) with similar sticky overhang at both ends, and telomeres (blue) with the telomeric G-rich overhang distally and a subtelomeric residue (light blue) containing the same sticky overhang as the genomic fragments, at the proximal end.*

3) *Two specially designed panhandle oligos (light and dark green) are ligated to the constructed sticky overhangs. This gives rise to genomic fragments with panhandle oligos in both ends, and telomeric regions with panhandle oligos at the proximal end.*
A Telorette-oligo (turquoise) is next ligated to the G-rich overhang of the telomeres.

4) *An initial step to the PCR reaction fills in the panhandle oligos, making them double stranded.*

5) *During PCR the genomic fragments form stable panhandles, due to the structure of the panhandle oligos ligated to both ends of the fragment. This structure has a high melting temperature, making the panhandle stable throughout the PCR-reactions, thereby suppressing amplification of the genomic fragments.*

6) *For the telomeres the fill in works for only the proximal, subtelomeric end. Here it creates a primer site for the upstream primer, the Adapter (pink), initiating amplification of the telomere.*
A Teltail primer (purple) will in following PCR-rounds be able to use the PCR-product initiated by the Adapter, as a primer site. This gives rise to telomere products of different lengths reflecting the telomeres in the sample analysed.

Modified from Aging Cell, Volume 9, issue 3, Laila Bendix, Peer Bendix Horn, Uffe Birk Jensen, Ivica Rubelj, Steen Kolvraa, "The load of short telomeres, estimated by a new method, Universal STELA, correlates with number of senescent cells", pages 383-397, Copyright © 2010 Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland, with permission from John Wiley and Sons. Figure legend is modified.

The Telorette chosen for the U-STELA method is assumed to cover about 80% of the telomeres. Additionally, the nature of the PCR is such that there is a stochastic choice of templates during the first few cycles. We therefore performed 8 separate PCR-reactions per patient sample to be analysed. The mean value from these eight repeats was used as an estimate of the amount of ultra-short telomeres in the sample.

We tested several PCR-settings. Several versions of elongation time were tested, as the number of shorter telomeres appeared to be quite high in our UC-samples. The PCR-products were separated using regular agarose gel electrophoresis in TBE (Tris-borate EDTA) buffer. TBE was chosen over TAE (Tris-acetate EDTA) because it has a lower risk of overheating during long gel electrophoresis, and also as it produces a better visualisation of smaller fragments. A U-STELA DNA separation runs for 2 hours, at 5 volts per gel cm, to ensure separation of fragments from 23 kb to fragments of less than 1 kb. Several percentages of gel were tested, along with several speeds for separation. The 0.85 % agarose gel was chosen as it gave good separation of the smaller fragments, and allowed for wandering of the longer fragments through the gel. 5V per gel cm was chosen to avoid too much heat to the gel.

Southern blot analysis is a rather lengthy transfer procedure from agarose gel to membrane. We thus transferred the fragments from agarose gel to membrane by vacuum (VacuGene XL Vacuum blotting Systems; GE Healthcare). This ensures a rapid transfer of fragments, cutting the total procedure time by hours. The use of a vacuum blotter also maintains similar conditions through repeated analyses, considerably reduces the amount of transfer buffer used by the procedure, and ensures a more even blotting than achieved using traditional capillary force blotting. For a Southern blot there is a choice of using nylon or nitrocellulose membrane. We chose to use a nylon membrane, as it is slightly more durable than the nitrocellulose one and

allows for permanent linking of the DNA fragments to the membrane by UV for half a minute in a Stratalinker UV crosslinker (Stratagene), rather than by baking the membrane at 120°C for 30 minutes. A noteworthy feature concerning the nylon membrane is that it binds any lipids, all through the procedure. Fingerprints on a developed U-STELA membrane do nothing to enhance the interpretation of the telomere bands. To avoid unnecessary stress on the membranes as well as excess use of reagents, and to ensure similarity between the membranes, we used hybridisation tubes.

Visualisation was performed by adding a digoxigenin (DIG)-labelled telomere probe (Roche) that binds to the telomere sequences embedded in the membrane. DIG can be visualized via chemiluminescence, using an anti-DIG antibody and an alkaline phosphatase substrate, thus avoiding radioactivity.

The U-STELA measures the amount of telomeres below a set threshold value. The issue of choosing a threshold value for counting telomeric bands provided some difficulties. UC-colonic mucosa is subjected to increased rates of proliferation, thus shortening the mean telomere length faster than can be expected of other tissue. Also, the mucosal cells in a UC-colon are exposed to elevated levels of oxidative stress that may cause abrupt shortening of one or a few telomeres. We initially set our threshold value at 1500 base pairs, but observed, in a high number of progressor cases, that the threshold value was covered by a smear of telomeric DNA rather than by discrete, separate bands. We therefore decided on a threshold level of 900 base pairs, as this was a length where most progressors displayed separate bands. Very few non-UC control samples displayed bands at this value. The separate bands visualised by the probe are considered to be single telomeres, amplified by the PCR. This required that we count any single band in the blot as a separate telomere, regardless of the strength of the signal.

By applying the estimation of a cell's amount of DNA (approximately 8pg) U-STELA can be used for estimating the amount of ultra-short telomeres from the genome equivalent of a single cell. The bands from a sample are counted, and the number divided by the genome equivalent of the DNA added to the PCR-reactions. However, some of our samples included aneuploid populations, of which the very definition is abnormal DNA-content. Indeed, most of the aneuploid populations in our aneuploid samples had a DNA-index of 1.5 (meaning they contained 50% more DNA in their cells than in normal diploid cells). The DNA-equivalent of these samples would therefore be more than 8pg, and estimations of the amount of ultra-short telomeres could thus be influenced by this factor. The most probable outcome of such an error would be an underestimation of the ultra-short telomeres within the aneuploid samples that would mask an association between ultra-short telomeres and development of aneuploidy. However, our samples were not sorted, and the aneuploid populations in the samples noted as aneuploid, comprised mostly 5-10 % of the total cell count as analysed by flow cytometry. Considering both these factors, we concluded that the amount of aneuploid cells in our aneuploid samples was not enough to contribute any substantial errors when estimating the amount of ultra-short telomeres. Rather, the high percentage of diploid cells in aneuploid samples could mask a possible association between aneuploidy and telomeres. We therefore decided not to calculate our results to a single cell level, and instead used our results to assess the load of ultra-short telomeres within a sample of colonic mucosal cells harvested from an area of either a progressor or a nonprogressor patient. Our aim concerning aneuploidy was to assess the load of ultra-short telomeres within an area harbouring an aneuploid population. To further examine a possible association between ultra-short telomeres and aneuploidy it would be necessary to sort cells by DNA-content to achieve a pure population of aneuploid cells prior to DNA extraction, or to use unsorted samples where aneuploid cells comprise more than 50% of the cellular population.

As the telomere bands were counted manually, the counting also presented a potential dilemma of bias when knowing if the case was a progressor or a non-progressor. This was accounted for by having several colleagues count the number of short telomeres in a blinded test.

The Universal STELA is a rather laborious method, demanding up to a full week before results can be obtained. When completed, it does not provide information on mean telomere length.

Because of the PCR-design, this method is biased towards the shortest telomeres in the sample analyzed. It can not be trusted to reveal the mean length of telomeres, as the amount of longer telomeres is suspected to be under-represented. This is probably due to insufficient amplification. Additionally, U-STELA cannot provide any information about the chromosomal origin of the shortened telomere.

However, we found it to be an elegant method of estimating the amount of ultra-short telomeres in a sample, thus providing information that is of real importance, and that would be lost if several other methods available for analysing telomeres in a complex sample had been used. In cases where chromosomal origin of the telomere is not considered to be of high importance this is a valuable method, to be performed preferentially in combination with a method providing mean telomere length, such as qPCR. This is what was done in Paper I.

qPCR to assess mean telomere length

Quantitative Polymerase Chain Reaction, qPCR, is a common method for analysing gene expression [194]. The qPCR based method of analysing mean telomeric length was presented in 2002 [186]. Several variants are later presented, to overcome methodological shortcomings of the initial method [179, 195, 196]. As is common with all PCR-based methods, it works using a minute amount of starting material and is easily applicable to large sample series. Telomere measurement by qPCR gives an estimation of mean telomeric length in a sample by analysing the amount of telomere sequence repeats (T) and comparing it to an internal control single copy gene (SCG). The ratio between T and SCG is then proportional to the mean telomere length for the sample analysed. The method is dependent on good quality DNA and concentration for accurate measurements.

To avoid pipetting errors, the samples were pipetted by the Biomek FX laboratory station. Choice of a single copy gene for the analysis provided us with a dilemma, as some of our samples included aneuploid populations. The standardised SCG established for the Danish Aging Research Center, University of Southern Denmark in Vejle, was Tert (Taq-Man Copy Number Reference Assay, Applied Biosystems, Denmark). Tert is located on chromosome arm 5p5.33, a region frequently amplified in aneuploid colorectal cancers [117, 161]. We therefore also conducted the analysis using RNAse P (TaqMan Copy Number Reference Assay, Applied Biosystems, Denmark) as another SCG. RNAse P is located on chromosome arm 14q11.2, which is not frequently altered in colorectal cancers [117, 161]. When the two SCGs were compared, the ratios were consistent between them, except for 6 lesions, all aneuploid, and four were adenocarcinomas. As RNAse P is located in a genomic area that is less affected by aneuploidisation, we used the T/SCG-ratio obtained from this analysis for our analyses of mean telomere length.

All samples were run in triplicates, and sample plates were run as independent duplicates. We registered an inter-assay coefficient of variation (CV) of 10.5% using the RNAse P as SCG, and a CV of 11.3% for TERT as SCG. The inter-assay CV value reports on the consistency between the plates. The values from our analyses are somewhat high, as the noted, typical CV for telomere length estimation by qPCR is 6-7% [197, 198]. This typical CV is however derived from a study on cultured leucocytes, and we can thus speculate that a pooled variety of colonic mucosal cells will give a slight increase in CV as compared to cultured cells. The elevated CV could also indicate a slight impurity in our samples, possibly due to low amount of starting material for DNA isolation or pipetting errors. Both are factors to consider when evaluating qPCR CV-values .It might also be an effect of long term storage of cells, as storage can influence CV-values for T/SCG-qPCR [199]. A potential source of elevated CV-values could also be in our use of the 7900HT Fast Real-Time PCR system, as this system has a plate position effect that might increase the CV-values.

Immunohistochemistry for hTERT, p53, and Aurora A proteins

Tissue preparation

Immunohistochemistry (IHC) facilitates the binding of a labelled antibody to the protein of interest. By combining anatomical, structural analysis with immunological techniques, the method can enable the localisation of protein expression in a tissue sample [200]. Quality immunohistochemical results are dependent on proper cell structure preservation. It is thus essential that the tissue of interest is rapidly preserved, either by freezing at -80°C, or by fixation in either ethanol or formalin (a ~40% solution of formaldehyde, diluted to 4%). This will ensure the preservation of morphological structures and prevention of tissue decay. Ethanol fixation leads to coagulation of tissue proteins, and produces a morphology that is not as well preserved compared to fixation in formalin. Preservation in formalin induces some molecular alterations, such as the formation of methylene ($-CH_2-$) bridges, or cross-links, between nucleic acids and proteins, and between proteins [201]. This cross-linking may mask the antigen epitopes by altering protein structures [202], thus antigen retrieval methods are needed to make the antigen available for antibody binding in subsequent immunohistochemical stainings.

Fixed tissue is further embedded in paraffin which enables long term storage of the sample. Embedding also facilitates easy sectioning of the tissue. For protein examination sections of 4-5 µm are cut using a microtome. Sections are mounted on microscope slides that are coated with an adhesive, such as poly-L-lysine. These slides can be stored at 4°C.

Our analyses of hTERT and Aurora A expressions were conducted on tissue micro arrays (TMAs), whereas p53 expression was assessed using whole sections of formalin-fixed paraffin-embedded (FFPE) tissue. Whole-section staining of p53 was decided upon because p53 staining has a heterogeneous distribution throughout tumour tissue (random areas of positivity and negativity). Tissue cores removed from these areas in a TMA could lead to possible scoring errors with an increase in false negatives if the scoring was done on TMA cores sampled from a negative area.

TMAs are made by taking core needle biopsies (usually with core size 0.6-3.0 mm in diameter) from a donor paraffin-embedded tissue block, and embedding them into a recipient paraffin block. Two or three cores from a donor block are sufficiently representative for analysis of protein expression in a tissue sample, comparable to a whole section of the entire tissue [203, 204]. Our TMAs consisted of at least three cores from each patient sample. The making of a TMA requires an experienced pathologist to evaluate the donor tissue sample and to mark the areas suitable for core sampling.

In performing IHC-analyses TMAs have certain advantages. The method facilitates the analysis of large tissue cohorts in a cost effective and time-saving manner. It also ensures identical staining and procedural conditions for the range of samples analysed. TMAs of colonic mucosa do not display the full colonic crypts, and this was cause for some concern when choosing section-material for protein expression analysis. However, both hTERT and Aurora A protein expression displayed sufficiently homogeneous distribution in stained whole sections for TMAs to be deemed suitable for expression analyses of these proteins. For our analyses, cells with positive nuclear staining were counted. For p53: at least 1000 cells chosen randomly were counted, and for the for the TMAs: at least 300 randomly chosen cells were counted from each core biopsy analysed.

Antibodies and antigen retrieval

hTERT immunohistochemistry has been shown to be somewhat troublesome, published reports have used antibodies that are no longer commercially available or even antibodies that have been shown to bind to other antigens [205]. For analysis of hTERT expression in our UC-colonic mucosal cells, a range of commercially available antibodies were tested, and any antibody that produced cytoplasmic staining was excluded. We found that mouse monoclonal ab518 (Abcam, Cambridge, UK), produced clear, nuclear staining with excellent reproducibility. It also resulted in the correct size protein on a western blot analysis: 127 kD. Antigen retrieval for tissue fixed in formalin includes heat exposure to destroy the cross-linking. This can be achieved using pressure cooking, autoclaving, steam heating or microwaving. In our lab microwaving is the method of choice for antigen retrieval, since it is an easily accessible method and allows for easy adjustment of heat exposure to accommodate examination of different proteins. Antigen retrieval of hTERT and p53 was conducted in citrate buffer. Tris-EDTA was used for antigen retrieval for Aurora A.

p53 immunohistochemistry were performed as is the established routine in our laboratory[132], using mouse monoclonal Ab-2 ((Pab 1801) Oncogene Research Products, Cambridge MA, USA). This antibody has produced consistently good results of nuclear staining.

Aurora A immunohistochemistry was performed using a mouse monoclonal antibody, NCL-L-AK2 (Novocastra, Newcastle, UK). This produced minimal background staining and a protein size on a western blot analysis at 46 kDa [206].

Mutation analyses for TP53

Several methods are available for the detection of point mutations and single base substitutions, deletions, or insertions. Choosing the appropriate method is dependent on a range of parameters, such as the kind of specimen to be analysed, size of the fragment, number of potential mutations in the sequence, and the suspected ratio between wild-type and mutated version of the alleles. The different methods used for detection are mainly based on the principles of heteroduplex DNA cleavage, or separation of double strand DNA fragments on denaturing gels. Mutations may also be identified by sequencing the PCR-products directly, however this may be complicated by the base-pairing error frequency of the polymerase used, and is not a suitable method if the target gene has few mutated alleles in an excess of wild-type alleles [207, 208].

Heteroduplexes are DNA strands containing a noncomplementary base pair. These may be generated by denaturation and subsequent reannealing of wild-type and mutant DNA fragments after PCR amplification. Homo- and heteroduplexes may then be distinguished by their migratory abilities through nondenaturing polyacrylamide gels by gel electrophoresis. Heteroduplexes may also be subjected to cleavage, and separation through denaturing gel electrophoresis. Cleavage of the fragments can be performed chemically, by enzymes, or by RNase. Mismatches in heteroduplexes may also be detected by mismatch binding protein association. The DNA fragments are then incubated with mismatch binding proteins. The bound and unbound DNA fragments' are then distinguishable by mobility shift electrophoresis [209]. These methods are generally limited by the amount of mutations detected. Chemical cleavage is generally more sensitive, but limited by the use of toxic substances [207].

Denaturing gradient or temperature gradient gel electrophoresis (DGGE or TGGE respectively), use an increasing chemical or thermal gradient with gel electrophoresis of double stranded DNA. Wild-type and mutated DNA fragments will display different migratory capability, due to differences in the melting temperature of the DNA strands. Mismatched base pairs will alter the melting temperature of the DNA fragment. The method can detect both wild-type and mutant homoduplexes, as well as heteroduplexes, but is limited by laborious optimisation and stringency of temperature [207, 208, 210]. Constant denaturant capillary electrophoresis (CDCE) uses polyacrylamide gel filled capillaries [211-213], thus making the method suitable for higher throughput analyses. The method is however still somewhat limited by stringent temperature control.

Cycling temperature capillary electrophoresis (CTCE)

By cycling the temperature several degrees around the calculated melting temperature of the sequence to be analysed, the wild-type and mutated sequences can be separated, without the need for stringent temperature control. This cycling temperature also reduces the amount of optimising needed, and facilitates the analysis of multiple target sequences in the same run [208].

We performed CTCE on exons 5-8 of *TP53*. Exons 5-8 are known mutational hotspots for point mutations in the *TP53* sequence. Further analyses should thus focus on the mutations outside these hotspots, and also on sequencing the mutations already detected.

Statistical considerations

Proper statistical handling of empirical data generated in an experiment is crucial to drawing valid, scientific conclusions. The field of biostatistics today is complicated, and dependent on powerful computer tools to investigate hypotheses and possible correlations. The complete understanding of biostatistics and statistical analysis of biomedical data is thus not possible for the individual researcher in general, and collaboration with biostatisticians is essential for many research projects.

In statistics a null hypothesis proposes what is assumed to be true. The null hypothesis assumes no difference between the variables tested, and needs a 95% certainty that the data do not comply with the hypothesis to be rendered false. Formulating the null hypothesis is an essential step in testing the statistical significance of a result; the possibility that a result did not happen by chance.

After formulation of the null hypothesis there comes the choice of statistical methods. Our data were obtained from a set of colectomies, all contributing with more than one biopsy. Multiple entries per subject analyzed will generally induce a set of errors following the natural variance between individuals. These interpatient variations could then mask significant associations between the parameters we aimed to study, or induce incorrect significances as our sample set included a limited set of patients.

To compensate for the repeated measurements several methods have been introduced. Most are limited by discarding all subjects with even one missing measurement, which would be rather disastrous for our studies as we operate with small sample sizes. The linear mixed model (LMM) will handle missing values by applying a “missing-at-random” definition, an invaluable point to our study of multiple biopsies from each patient, with not all being full sets of eight measurements for all parameters. LMM also incorporates both fixed and random effects, giving the result as the sum of these effects. A limitation to the method is that it is bound to the use of mean values, where median values could sometime be preferential to analyzing smaller sample sizes. The use of mean or median is usually dependent on the data distributions, thus evaluation of whether the data presents as Gaussian (normal) or non-Gaussian distributions.

In the first paper the material was limited to only a subset of the 30 patients, and we started by using non-parametric t-tests and one-way ANOVA to examine the relationship of telomere data to the morphological traits investigated. LMM was introduced when we combined fixed and random effects for the main analysis of distribution differences of telomere data between progressors and nonprogressors. The p-values were still significant. LMM was thus the preferred method throughout our studies.

In paper II we also applied cross tabulation analysis and Pearson’s Chi-square test to assess any correlation of *TP53* mutation status and p53 protein positivity within the progressors. Both factors are reported in a categorical, dichotomous manner and a cross tabulation is a simple way to investigate any interaction between two survey points.

Summary of results

Paper I:

Telomere shortening correlates to dysplasia but not to DNA aneuploidy in longstanding ulcerative colitis

Ulcerative colitis patients have an elevated risk of developing colon cancer, and the risk factors are still not fully known. There is also no current method of distinguishing a patient who will progress to dysplasia and cancer from one who will not. The colonic mucosa of ulcerative colitis patients is subjected to rapid cell turnover and elevated levels of oxidative stress. Both can induce rapid shortening of telomeres. Telomere shortening is associated with both dysplastic development and the induction of chromosomal instability (CIN) and aneuploidy. However, it has been suggested that it is not the bulk shortening of telomeres that may induce CIN and aneuploidy, but rather the shortening of one or a few telomeres: ultra-short telomeres.

It would then be of interest to examine the distribution of the shortest telomeres in a sample, in addition to the mean telomere length. We applied a rather novel method designed for assessing the amount of ultra-short telomeres: the Universal STELA, to samples collected from a set of progressor and nonprogressor UC colectomies. The amount of ultra-short telomeres was measured along with mean telomere length using qPCR. We then assessed the results between progressors and nonprogressors. Within the progressors we also compared aneuploid lesions to diploid lesions, as well as dysplastic lesions to non-dysplastic lesions.

We found that the amount of ultra-short telomeres was significantly elevated in progressors compared to nonprogressors. Mean telomere length did not differ between the two groups. Within the progressor cases mean telomere length was reduced in dysplastic mucosa, whereas the amount of ultra-short telomeres was increased. We found that telomere shortening did not associate with aneuploidy in our samples.

Based on our findings, we suggest that the amount of ultra-short telomeres might be a possible biomarker for detection of a UC-progressor.

Paper II:

TP53/p53 alterations and Aurora A expression in progressor and nonprogressor colectomies from patients with longstanding ulcerative colitis.

Aneuploidy is assumed to be caused by missegregation of chromosomes during mitosis, often due to a faulty spindle assembly checkpoint. p53 is a tumour suppressor protein known to regulate the spindle assembly checkpoint, and is frequently mutated in aneuploid cells. Aurora A is a presumed oncoprotein, also involved in regulation of the spindle assembly checkpoint. A negative regulatory feedback loop involving p53 and Aurora A has been reported. In this study we examined the mutational frequency of *TP53*, and the p53 positivity and Aurora A expression in UC progressors and nonprogressors. Within the progressors we further examined the expression of Aurora A in association with mucosal morphology and aneuploidy.

We did not detect any correlation between *TP53* mutations and p53 positivity, and p53 positivity was only detected within the progressor colons. *TP53* mutations were detected in both progressors and nonprogressors. Furthermore, we did not observe any differences in Aurora A expression between the progressors and nonprogressors. These results may indicate the presence of a functional p53, and thus possibly a senescent protection mechanism in the nonprogressors. The presence of *TP53* mutations in areas revealing no p53 positivity may be indicative of a mutation prior to development of LOH.

Within the progressors we detected decreased p53 levels in lesions harbouring aneuploid populations. Aurora A levels decreased with increasing severity of dysplasia in the mucosal morphology and with aneuploidy. Lesions positive for p53 also displayed decreased levels of Aurora A. All adenocarcinomas displayed elevated levels of Aurora A, in accordance with previous publications. It is possible that elevated levels of Aurora A expression may be involved in a protective mechanism, such as oncogene-induced senescence. The findings of decreased Aurora A levels in lesions positive for p53 are consistent with a scenario where only wild-type p53 regulates Aurora A.

The study suggests that Aurora A is not a suitable biomarker for detection of a progressor, but that p53 expression may be promising in the search for biomarkers identifying a UC-progressor.

Paper III:

Reduced hTERT protein levels are associated with DNA aneuploidy in the colonic mucosa of patients suffering from longstanding ulcerative colitis.

Telomerase activity is frequently reported in cancerous cells, but is deactivated in most healthy, somatic cells. Activation of telomerase can enable replicative immortality, resulting in an accumulation of genetic alterations, which in turn can contribute to the development of cancerous cells. In UC reduced as well as increased telomerase activity have been reported. hTERT, the catalytic subunit of telomerase, is suggested to be the limiting factor for telomerase assembly and thus function. In this work, we applied immunohistochemistry (IHC) to examine the levels of hTERT within a set of progressor and nonprogressor UC colectomies. Protein levels in the progressors were also examined in association with aneuploidy and advancing stages of dysplasia; cancerous lesions were excluded. hTERT levels were also assessed in association with the levels of the proliferation marker Ki67.

Protein levels of hTERT did not differ between progressors and nonprogressors. Within the progressors we did neither notice any association between hTERT levels and ploidy-status, nor between increasing degrees of dysplasia. However, within each of the advancing degrees of dysplasia aneuploid lesions showed less hTERT expression than diploid lesions, indicating that lower levels of hTERT are associated with aneuploidy, when the confounding factor of mucosal morphology is accounted for. We also detected an association between hTERT levels and Ki67 expression within the nonprogressors. This association was lost in the progressors, even when including only the diploid, non-dysplastic progressor lesions. This may indicate that the association is a possible protective mechanism against the development of malignant traits in a UC-colon.

General discussion

This chapter presents an integrated discussion of the results in relation to the aims of this thesis; specific discussions of the individual results of each study are provided in the specific articles.

Our initial idea for this project was to examine telomeric differences between sorted aneuploid and diploid populations of sporadic colorectal cancers. Following manual disaggregation of sporadic colorectal tumours and isolation of nuclei for DNA flow cytometry, the nuclei were sorted using a FACS DiVa flow cytometer. DNA isolated from the sorted nuclei was unfortunately not suitable for U-STELA analyses, regardless of the method used to retrieve DNA. Due to this problem, areas containing aneuploid and diploid populations from ulcerative colitis colectomies were instead chosen for U-STELA-analysis. These colectomy samples were resected in the 1980s and 90s, thus some cell-suspensions were lacking, due to previous analyses that had utilized and depleted some patient samples.

Initial analyses in Article I compared aneuploid to diploid, and dysplastic to non-dysplastic lesions from UC-colectomies. During these analyses we did not consider progressors and nonprogressors, and all lesions included in our initial tests were considered as separate, independent entries. However, the material used for this study comprises colectomies all yielding at least eight lesions, and curiosity as to whether the amount of ultra-short telomeres was dependent on colonic location spurred the analysis of all eight lesions from colons including both diploid and aneuploid lesions, of dysplastic and non-

dysplastic morphology. As controls we included lesions from colectomies where no dysplastic lesions had been reported upon examination by two experienced pathologists. This separation of colectomies based on dysplastic traits is also seen in the UC-literature, introducing us to the terms progressors and nonprogressors [18, 90, 214].

Advice from a statistician made us aware that our initial analysis, treating all included lesions as independent entries, might result in patient differences that could perhaps mask genuine effects. We thus conducted linear mixed model analyses (LMM), where patient differences were accounted for. As the results seen by our initial analyses were still valid, they were published in the first article included in this thesis, together with results of the LMM.

For Articles II and III, the aim was to investigate selected proteins associated with telomere regulation and cell division, using IHC on formalin fixed patient material. Both articles include a set of 20 progressors and 10 nonprogressors.

In Article II, that reported our findings of Aurora A and p53, we found a significant decrease in the protein levels of Aurora A that was associated with increasing degrees of dysplasia, followed by an increase in the Aurora A levels in the adenocarcinomas. This inspired a separate analysis where we divided the colectomies by the presence or absence of an adenocarcinoma. Article III reported our findings on hTERT-levels. No differences were detected in hTERT levels between precancerous and cancerous lesions. We therefore found it more interesting to analyse the pre-cancerous lesions from the included UC-colectomies, excluding the adenocarcinomas.

All patients suffering from UC are subjected to recurring periods of active inflammation and can thus be assumed to endure elevated levels of oxidative stress to the colonic environment. None the less, only some progress to presenting DNA aneuploidy, dysplasia, or to the utmost consequence: adenocarcinoma.

Classifying of progressors and nonprogressors is a way of distinguishing patients suffering from UC, based on the detection of dysplastic changes or not [18]. In our investigations we also included the detection of aneuploid populations as a progressor trait, as it is acknowledged as a precursor for malignancies in UC [19, 215]. This additional trait did not add any patients to the progressors on behalf of the nonprogressors, as we found that none of the colectomies harbouring aneuploid areas were void of any dysplasia. Aneuploid populations were detected in dysplastic as well as non-dysplastic areas, and colons presenting areas with aneuploid populations and displaying but one lesion indefinite for dysplasia were thus considered progressors.

All UC patients theoretically endure the same prerequisites for advancing to progressors, as they all experience extensive periods of active inflammation. However, the fact is that only a subset of patients with longstanding UC, estimated to about 10 %, will develop dysplasia. It is suggested that nonprogressors may have a more functional DNA damage repair system compared to progressors [32, 164]. Also: overexpression of hTERT has been shown to enhance antioxidant defence mechanisms against oxidative stress-induced damage in cancer cells [164, 216, 217]. Perhaps elevated telomerase activity levels might render nonprogressors less prone to oxidative stress-induced DNA-damage. Given the multifactorial pathology of UC, with the implications of molecular changes, immune responses and probable genetic susceptibilities [2, 10, 17, 218], it is suspected that individual patient differences will account for whether a patient will progress or not.

However, as statistical analyses would then be made virtually impossible by the numbers of patient categories, the collective terms progressors and nonprogressors were used. Our studies focused on elucidating similarities and differences between patients grouped according to these two terms.

We found that progressors and nonprogressors revealed similar levels of mean telomere shortening, and that hTERT and Aurora A protein levels were equally elevated (apart from individual differences as mentioned above) between the two groups. These results allow us to speculate whether both progressors and nonprogressors endure similar telomere attrition. All patients had endured UC for more than 10 years, a probable explanation for the lack of difference between the two groups [87]. The main differences detected were significantly elevated levels of ultra-short telomeres and also areas of p53 positivity in the progressors.

The enhanced levels of Aurora A in combination with no detectable p53 accumulation throughout the nonprogressors may be interpreted as upregulated SAC activity, possibly due to enhanced proliferation that is known to occur in UC-colonic mucosa [85, 86]. Most nonprogressors will thus probably possess an intact spindle assembly checkpoint (SAC). If checkpoints are intact, any telomere shortening past a certain point will most likely be detected as an anomaly and the cell will be sentenced to a senescent state by pathways involving p53, in the telomeric induced senescence. This enhanced proliferation rate may at the same time contribute to an upregulation of the SAC activity and Aurora A expression. As an additional protective mechanism of the nonprogressor, the detected, elevated levels of hTERT may then be active in a non-telomeric mechanism, strengthening the cells antioxidant defence ability against oxidative damage.

Lack of correlations between mutation of *TP53* and detected protein accumulation of p53 has been shown for UC-progressors previously [219]. Mutations in *TP53* are proposed as an early marker for malignant development in UC, whereas p53 loss of heterozygosity (LOH) may be a later trait more closely associated with DNA aneuploidy [144, 220]. We find our results of detected *TP53* mutations without correlation to detectable p53 positivity in the progressors, as well as *TP53* mutations detected in the nonprogressors where no p53 positivity was registered, consistent with these studies.

Our analyses comprised mutations in the exons 5-8. We noticed that the majority of mutations detected in the nonprogressors were in exon 5, whereas all examined mutational sites were detected in the progressors, with no discernible pattern emerging (unpublished data). These data lead us to speculate as to the types of mutations in exon 5 (missense or nonsense) and their impact on p53 accumulation. This could be elucidated by extended analyses and also sequencing. Unfortunately these extended analyses were not performed in our studies.

Overall, the progressors differed from the nonprogressors by two of the parameters investigated in this thesis; the load of ultra-short telomeres was significantly elevated in the progressors, and accumulation of p53 was detected in a percentage of the lesions examined. Together, these results could support the theory of a better DNA-repair function and defence against oxidative damage in the nonprogressors, as also suggested by others [32]. In the overall comparison of progressors and nonprogressors, we detected no difference in hTERT levels, which perhaps would not support the position of hTERT as an antioxidant strengthening effect.

However, the progressors will comprise a more heterogeneous set of lesions than the nonprogressors, since a lesion may be aneuploid or diploid as well as dysplastic or non-dysplastic. Also adenocarcinomas may present a completely different biology. All of these factors need to be considered, and the lesions should therefore be analysed as separate categories within progressors. The progressor lesions were thus divided according to morphologic as well as DNA-ploidy related differences. All analyses also included the overall possibility of patient variation.

The diploid, non-dysplastic lesions of the progressors differed from those of the nonprogressors with regards to the amount of ultra-short telomeres. The progressor lesions that should, in theory, not differ from the nonprogressors had statistically significant increased numbers of these critically shortened telomeres. Mean telomere length, hTERT levels, and Aurora A levels did not present any differences when comparing the diploid, non-dysplastic progressor lesions to those of the nonprogressors.

The amount of ultra-short telomeres did not differ between the separate parameters investigated within the progressors. We thus speculate that differences in amount of ultra-short telomeres as detected by U-STELA between progressors and nonprogressors may be an indication that the progressors endure more stress to the cellular environment than the nonprogressors, even if the lesion investigated is diploid and not presenting any dysplasia. The lesions included in the category of diploid, non-dysplastic progressor areas included lesions from colectomies with one single dysplastic area to colons harbouring only one, single non-dysplastic area. Also, origin of the lesion, with respect to distance from dysplasia, was not taken into consideration. Differences in molecular biology between non-dysplastic lesions distal and proximal to a dysplasia have been reported [164]. The progressors also varied in the amount of aneuploidisation through the colon.

This may also be a contributing factor to the amount of ultra-short telomeres detected in the diploid, non-dysplastic lesions, as pancolonic genomic instability and aneuploidy have been reported prior to dysplasia and cancer in UC [47, 48, 56, 141, 221, 222].

Within the progressors included in our studies, aneuploid lesions were associated with lower levels of Aurora A and hTERT compared to diploid lesions, without any effect detected within the telomeric parameters examined. The lesions containing aneuploid populations also contain diploid cells. In most of our aneuploid samples the diploid cells accounted for the majority of the sample. This may mask any differences in telomere parameters between aneuploid and diploid populations, especially concerning the results seen in telomere shortening. It can thus be speculated that any changes associated with difference in DNA-ploidy status, detected in the areas examined are early alterations as it was most likely detected in the diploid DNA, as well as the aneuploid DNA. It is then possible that lowered levels of Aurora A and hTERT are implicated in the initiation of field aneuploidisation. As hTERT levels in diploid non-dysplasia were not statistically distinguishable from nonprogressors, and levels were decreased in aneuploid lesions compared to diploid lesions of all degrees of dysplasia examined. We also detected higher levels of hTERT expression in progressor colons not harbouring adenocarcinomas, compared to progressors with adenocarcinomas, yet to no statistically significant degree (unpublished data). These findings may support the possibility of elevated levels of hTERT acting as a protective mechanism against damage caused by excess oxidative stress [216].

As aneuploidy can develop through CIN and BFB-cycles, an additional speculation may be that telomere bridging and rearranged chromosomes can mask formation of even more ultra-short telomeres. The amount of the ultra-short telomeres detected in progressor lesions can then enable further rearrangement and BFB-cycling. The decreased levels of Aurora A might point to a decreased activity in the SAC-complex. All may be implicated in the development of an aneuploid population. A causative effect, however, can not be determined from our retrospective study using archival material.

Dysplasia may be divided according to degree of severity, and UC-related colon cancer is though to develop in a multistep fashion through advancing degrees of dysplasia. We divided the dysplastic lesions of our material according to degree of dysplasia, and assessed the status of each parameter analysed accordingly. Based on this it seems that increasing degrees of dysplasia have similar amounts of ultra-short telomeres, decreased mean telomere length, and decreased levels of Aurora A. hTERT seem to associate more with DNA-ploidy status than with dysplastic development. We did not assess the potential impact of location of the dysplastic lesions through the colon, apart from a preliminary analysis showing no difference in any of the parameters studied through the locations within a colon (unpublished data). We thus speculate that dysplasia may develop though shortening of mean telomere length and a non-functional SAC. The amount of ultra-short telomeres seems to be generally elevated in the progressors, regardless of degree of dysplasia and DNA-ploidy status. This may indicate that ultra-short telomeres can be a driver of progression by rearranging the genome through telomeric ends too short for assembly of the telomeric T-loop and the shelterin proteins. A protective mechanism through elevated hTERT expression as detected in the diploid lesions might be suggested for UC-colonic mucosal cells, as we detected elevated levels in diploid lesions of all dysplastic degrees, regardless of other parameters measured.

The differences detected between progressors and nonprogressors by U-STELA suggest that the amount of ultra-short telomeres may be a promising biomarker for identification of a UC-progressor colon. This suggestion is further supported by the lack of differences detected within the parameters studied in the progressors, indicating a certain robustness of the amount of ultra-short telomeres as a potential biomarker, and of the U-STELA method in detecting a progressor.

Conclusions and future perspectives

In paper I we reported that the amount of ultra-short telomeres detected in the colonic mucosa of progressors is significantly elevated compared to that of nonprogressors, whereas no difference was detected in the mean telomere length of the colonic mucosa between these two groups of UC patients. We thus suggest that the amount of ultra-short telomeres may be a useful biomarker of a UC-progressor.

In paper II we reported that p53 positivity detected by IHC was observed in progressors alone, whereas *TP53* gene mutations were detected in both progressors and nonprogressors. Aurora A protein levels were decreased in lesions harbouring aneuploid populations as well as in dysplastic lesions, and increased significantly in adenocarcinomas. From this we speculate if elevated levels of Aurora A indicate elevated SAC-activity, or if it may be implicated in oncogene induced senescence. We also suggest that p53 protein accumulation may be a potential biomarker of a UC-progressor.

In paper III we reported that protein levels of hTERT were associated with the presence of aneuploid populations in the progressors, and that no difference in hTERT levels was seen between the diploid, non-dysplastic lesions of progressors, compared to the areas of the nonprogressors. Furthermore, an association between hTERT levels and the proliferation marker Ki67 was seen within the nonprogressors only. We thus speculate that an association between hTERT and Ki67 may have a protective effect against malignant development. We also speculate if elevated levels of hTERT may be involved in the defence against damage caused by oxidative stress.

Future studies should continue investigations of the amount of ultra short telomeres. Our analyses were somewhat limited by the sample size. If the amount of ultra-short telomeres is to be used as a biomarker for progressor colons, a study with a larger sample size is needed to confirm the significant associations detected. It might also be of interest to test the U-STELA method using blood samples. If differences in the load of ultra-short telomeres could be detected in blood samples of UC-patients, unnecessary invasive procedures to obtain colon biopsies could be avoided in the future.

Future studies should use the U-STELA method on sorted aneuploid and diploid UC-populations, or on a set of unsorted aneuploid lesions that contain more than 50% aneuploid cells. We would also like to expand the sample size to include a set of patients with disease onset past 50 years old, as well as a set of patients with disease duration less than ten years. It has been shown that disease traits may be different in patients developing UC after the age of 50, compared to patients with UC developed during young adulthood [223, 224], and that telomere attrition occurs mainly within the initial decade [87, 88].

Further analyses of telomeres and telomere-associated biomarkers, should also include studies of DNA damage, through examination of phosphorylation of histone H2AX. In its phosphorylated form, γ-H2AX, it is a biomarker for DNA-strand breaks, and has been reported to increase in senescent cells [225]. As a continuation of the hTERT-analyses, sequencing *hTERT* from nonprogressors as well as in the different lesions of the progressors, would be interesting to investigate if splice site differences can be detected and further investigated for a role in the progression towards malignancies [226]. Extended analyses of the mutations detected in *TP53*, to investigate the possibility of mutations in exons other than the ones included in this study would also be interesting. It may be valuable to sequence the mutations detected, in order to examine whether nonprogressors and progressors harbour different types of *TP53* mutations.

Senescence has been identified as a potent preventive mechanism against malignant development, and is induced by several pathways. Both telomere shortening and oncogenes are identified as possible inducers of senescence. We found that the amounts of ultra-short telomeres are elevated in nonprogressors compared to non-UC colon mucosal cells, and even more ultra-short telomeres are present in the progressors. From this we speculate if senescence might be a protective mechanism present in the nonprogressors. To investigate this possibility we aim to examine the protein expression of possible senescence markers by IHC. Candidate genes of interest are at present the Insulin-like growth factor-binding protein 7 (IGFBP7), a senescence associated secreted factor, which seems to be associated with escape of p53-induced senescence in sporadic colon cancers [227], and Senescence-Associated β-Galactosidase (SA-β-Gal), the current, most common marker for senescence, and also readily detectable by IHC analyses [228]. In addition to separately analysing these candidate genes, we would like to perform combination analyses, as we believe a possible biomarker may lie in network differences within the progressor and nonprogressor UC-colons.

References

1. Danese, S. and C. Fiocchi, *Ulcerative colitis.* N Engl J Med, 2011. **365**(18): p. 1713-25.

2. Baumgart, D.C. and S.R. Carding, *Inflammatory bowel disease: cause and immunobiology.* Lancet, 2007. **369**(9573): p. 1627-1640.

3. Aamodt, G., M.B. Bengtson, and M.H. Vatn, *Can temperature explain the latitudinal gradient of ulcerative colitis? Cohort of Norway.* BMC Public Health, 2013. **13**: p. 530.

4. Cosnes, J., et al., *Epidemiology and natural history of inflammatory bowel diseases.* Gastroenterology, 2011. **140**(6): p. 1785-94.

5. Ahuja, V. and R.K. Tandon, *Inflammatory bowel disease in the Asia-Pacific area: a comparison with developed countries and regional differences.* J Dig Dis, 2010. **11**(3): p. 134-47.

6. Langan, R.C., et al., *Ulcerative colitis: diagnosis and treatment.* Am Fam Physician, 2007. **76**(9): p. 1323-30.

7. Baumgart, D.C. and W.J. Sandborn, *Inflammatory bowel disease: clinical aspects and established and evolving therapies.* Lancet, 2007. **369**(9573): p. 1641-57.

8. Di Sabatino, A., et al., *Recent advances in understanding ulcerative colitis.* Intern Emerg Med, 2012. **7**(2): p. 103-11.

9. Eaden, J.A., K.R. Abrams, and J.F. Mayberry, *The risk of colorectal cancer in ulcerative colitis: a meta-analysis.* Gut, 2001. **48**(4): p. 526-35.

10. Ullman, T.A. and S.H. Itzkowitz, *Intestinal inflammation and cancer.* Gastroenterology, 2011. **140**(6): p. 1807-16.

11. Itzkowitz, S.H. and N. Harpaz, *Diagnosis and management of dysplasia in patients with inflammatory bowel diseases.* Gastroenterology, 2004. **126**(6): p. 1634-1648.

12. Eaden, J.A., et al., *Guidelines for screening and surveillance of asymptomatic colorectal cancer in patients with inflammatory bowel disease.* Gut, 2002. **51 Suppl 5**: p. V10-2.

13. Sunkara, S., et al., *Chronic Inflammation and Malignancy in Ulcerative Colitis.* Ulcers, 2011. **2011**.

14. Chambers, W.M., et al., *Cancer surveillance in ulcerative colitis.* Br J Surg, 2005. **92**(8): p. 928-36.

15. Macdougall, I.P., *The Cancer Risk in Ulcerative Colitis.* Lancet, 1964. **2**(7361): p. 655-8.

16. Stenoien, D.L., et al., *Dynamic association of a tumor amplified kinase, Aurora-A, with the centrosome and mitotic spindle.* Cell Motil Cytoskeleton, 2003. **55**(2): p. 134-46.

17. Thorsteinsdottir, S., et al., *Pathogenesis and biomarkers of carcinogenesis in ulcerative colitis.* Nat.Rev.Gastroenterol.Hepatol., 2011.

18. Ullman, T., et al., *Progression of flat low-grade dysplasia to advanced neoplasia in patients with ulcerative colitis.* Gastroenterology, 2003. **125**(5): p. 1311-9.

19. Rubin, C.E., et al., *DNA aneuploidy in colonic biopsies predicts future development of dysplasia in ulcerative colitis.* Gastroenterology, 1992. **103**(5): p. 1611-1620.

20. Maloy, K.J. and F. Powrie, *Intestinal homeostasis and its breakdown in inflammatory bowel disease.* Nature, 2011. **474**(7351): p. 298-306.

21. Wallace, K.L., et al., *Immunopathology of inflammatory bowel disease.* World J Gastroenterol, 2014. **20**(1): p. 6-21.

22. Kurashima, Y., Y. Goto, and H. Kiyono, *Mucosal innate immune cells regulate both gut homeostasis and intestinal inflammation.* European Journal of Immunology, 2013. **43**(12): p. 3108-3115.

23. Medzhitov, R., *Origin and physiological roles of inflammation.* Nature, 2008. **454**(7203): p. 428-35.

24. Grivennikov, S.I., *Inflammation and colorectal cancer: colitis-associated neoplasia.* Semin Immunopathol, 2013. **35**(2): p. 229-44.

25. Roessner, A., et al., *Oxidative stress in ulcerative colitis-associated carcinogenesis.* Pathol Res Pract, 2008. **204**(7): p. 511-24.

26. Hussain, S.P., L.J. Hofseth, and C.C. Harris, *Radical causes of cancer.* Nat Rev Cancer, 2003. **3**(4): p. 276-85.

27. Shackelford, R.E., W.K. Kaufmann, and R.S. Paules, *Oxidative stress and cell cycle checkpoint function.* Free Radic Biol Med, 2000. **28**(9): p. 1387-404.

28. Opara, E.C., *Oxidative stress.* Dis Mon, 2006. **52**(5): p. 183-98.

29. Waris, G. and H. Ahsan, *Reactive oxygen species: role in the development of cancer and various chronic conditions.* J Carcinog, 2006. **5**: p. 14.

30. Buffinton, G.D. and W.F. Doe, *Depleted mucosal antioxidant defences in inflammatory bowel disease.* Free Radic Biol Med, 1995. **19**(6): p. 911-8.

31. Seril, D.N., et al., *Oxidative stress and ulcerative colitis-associated carcinogenesis: studies in humans and animal models.* Carcinogenesis, 2003. **24**(3): p. 353-362.

32. Brentnall, T.A., et al., *Proteins That Underlie Neoplastic Progression of Ulcerative Colitis.* Proteomics Clin Appl, 2009. **3**(11): p. 1326.

33. Barzilai, A. and K. Yamamoto, *DNA damage responses to oxidative stress.* DNA Repair (Amst), 2004. **3**(8-9): p. 1109-15.

34. D'Angiolella, V., C. Santarpia, and D. Grieco, *Oxidative stress overrides the spindle checkpoint.* Cell Cycle, 2007. **6**(5): p. 576-9.

35. Thompson, S.L. and D.A. Compton, *Examining the link between chromosomal instability and aneuploidy in human cells.* J Cell Biol, 2008. **180**(4): p. 665-72.

36. Limoli, C.L. and E. Giedzinski, *Induction of chromosomal instability by chronic oxidative stress.* Neoplasia, 2003. **5**(4): p. 339-46.

37. von Zglinicki, T., *Oxidative stress shortens telomeres.* Trends in Biochemical Sciences, 2002. **27**(7): p. 339-344.

38. Gordon, D.J., B. Resio, and D. Pellman, *Causes and consequences of aneuploidy in cancer.* Nat Rev Genet, 2012. **13**(3): p. 189-203.

39. Holland, A.J. and D.W. Cleveland, *Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis.* Nat Rev Mol Cell Biol, 2009. **10**(7): p. 478-87.

40. Londono-Vallejo, J.A., *Telomere instability and cancer.* Biochimie, 2008. **90**(1): p. 73-82.

41. Cheung, A.L. and W. Deng, *Telomere dysfunction, genome instability and cancer.* Front Biosci., 2008. **13**: p. 2075-2090.

42. Kong, C.M., X.W. Lee, and X. Wang, *Telomere shortening in human diseases.* FEBS J, 2013. **280**(14): p. 3180-93.

43. Kops, G.J., B.A. Weaver, and D.W. Cleveland, *On the road to cancer: aneuploidy and the mitotic checkpoint.* Nat Rev Cancer, 2005. **5**(10): p. 773-85.

44. Bharadwaj, R. and H. Yu, *The spindle checkpoint, aneuploidy, and cancer.* Oncogene, 2004. **23**(11): p. 2016-27.

45. Janssen, A., et al., *Chromosome segregation errors as a cause of DNA damage and structural chromosome aberrations.* Science, 2011. **333**(6051): p. 1895-8.

46. Rajagopalan, H. and C. Lengauer, *Aneuploidy and cancer.* Nature, 2004. **432**(7015): p. 338-41.

47. Willenbucher, R.F., et al., *Genomic instability is an early event during the progression pathway of ulcerative-colitis-related neoplasia.* Am J Pathol, 1999. **154**(6): p. 1825-30.

48. Rabinovitch, P.S., et al., *Pancolonic chromosomal instability precedes dysplasia and cancer in ulcerative colitis.* Cancer Res., 1999. **59**(20): p. 5148-5153.

49. Brentnall, T.A., *Molecular underpinnings of cancer in ulcerative colitis.* Curr Opin Gastroenterol, 2003. **19**(1): p. 64-8.

50. Gerling, M., et al., *Aneuploidy-associated gene expression signatures characterize malignant transformation in ulcerative colitis.* Inflamm Bowel Dis, 2013. **19**(4): p. 691-703.

51. Gerling, M., et al., *High Frequency of Aneuploidy Defines Ulcerative Colitis-Associated Carcinomas: A Comparative Prognostic Study to Sporadic Colorectal Carcinomas.* Ann Surg, 2010. **252**(1): p. 74-83.

52. Meling, G.I., et al., *Flow cytometric DNA ploidy pattern in dysplastic mucosa, and in primary and metastatic carcinomas in patients with longstanding ulcerative colitis.* Br J Cancer, 1991. **64**(2): p. 339-44.

53. Lengauer, C., K.W. Kinzler, and B. Vogelstein, *Genetic instabilities in human cancers.* Nature, 1998. **396**(6712): p. 643-9.

54. Davoli, T. and T. de Lange, *The causes and consequences of polyploidy in normal development and cancer.* Annu Rev Cell Dev Biol, 2011. **27**: p. 585-610.

55. Meyer, K.F., et al., *Aneuploidy characterizes adjacent non-malignant mucosa of ulcerative colitis-associated but not sporadic colorectal carcinomas: a matched-pair analysis.* Scand J Gastroenterol, 2013. **48**(6): p. 679-87.

56. Lai, L.A., et al., *Pan-colonic field defects are detected by CGH in the colons of UC patients with dysplasia/cancer.* Cancer Lett, 2012. **320**(2): p. 180-8.

57. Itzkowitz, S.H. and X. Yio, *Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation.* Am J Physiol Gastrointest Liver Physiol, 2004. **287**(1): p. G7-17.

58. Hammarberg, C., P. Slezak, and B. Tribukait, *Early detection of malignancy in ulcerative colitis. A flow-cytometric DNA study.* Cancer, 1984. **53**(2): p. 291-5.

59. Harley, C.B., A.B. Futcher, and C.W. Greider, *Telomeres shorten during ageing of human fibroblasts.* Nature, 1990. **345**(6274): p. 458-60.

60. Allshire, R.C., M. Dempster, and N.D. Hastie, *Human telomeres contain at least three types of G-rich repeat distributed non-randomly.* Nucleic Acids Res, 1989. **17**(12): p. 4611-27.

61. Okuda, K., et al., *Telomere length in the newborn.* Pediatr Res, 2002. **52**(3): p. 377-81.

62. Griffith, J.D., et al., *Mammalian telomeres end in a large duplex loop.* Cell, 1999. **97**(4): p. 503-14.

63. Stewart, J.L., K.M. Baird, and C.J. Farr, *All's well that ends well.* Trends Cell Biol., 2001. **11**(7): p. 279-280.

64. de Lange, T., *T-loops and the origin of telomeres.* Nat Rev Mol Cell Biol, 2004. **5**(4): p. 323-9.

65. de Lange, T., *How telomeres solve the end-protection problem.* Science, 2009. **326**(5955): p. 948-52.

66. Hayflick, L. and P.S. Moorhead, *The serial cultivation of human diploid cell strains.* Exp Cell Res, 1961. **25**: p. 585-621.

67. Kuilman, T., et al., *The essence of senescence.* Genes Dev, 2010. **24**(22): p. 2463-79.

68. Wright, W.E. and J.W. Shay, *The two-stage mechanism controlling cellular senescence and immortalization.* Exp Gerontol, 1992. **27**(4): p. 383-9.

69. Maser, R.S. and R.A. DePinho, *Connecting chromosomes, crisis, and cancer.* Science, 2002. **297**(5581): p. 565-9.

70. Stewart, S.A. and R.A. Weinberg, *Telomeres: cancer to human aging.* Annu.Rev.Cell Dev.Biol., 2006. **22**: p. 531-557.

71. Shay, J.W. and W.E. Wright, *Senescence and immortalization: role of telomeres and telomerase.* Carcinogenesis, 2005. **26**(5): p. 867-74.

72. Shay, J.W. and W.E. Wright, *Telomeres and telomerase in normal and cancer stem cells.* FEBS Lett., 2010. **584**(17): p. 3819-3825.

73. Murnane, J.P., et al., *Telomere dynamics in an immortal human cell line.* EMBO J, 1994. **13**(20): p. 4953-62.

74. Hemann, M.T., et al., *The Shortest Telomere, Not Average Telomere Length, Is Critical for Cell Viability and Chromosome Stability.* Cell, 2001. **107**(1): p. 67-77.

75. Tusell, L., et al., *The number of dysfunctional telomeres in a cell: one amplifies; more than one translocate.* Cytogenet.Genome Res., 2008. **122**(3-4): p. 315-325.

76. Abdallah, P., et al., *A two-step model for senescence triggered by a single critically short telomere.* Nat Cell Biol, 2009. **11**(8): p. 988-93.

77. Murnane, J.P., *Telomeres and chromosome instability.* DNA Repair (Amst), 2006. **5**(9-10): p. 1082-1092.

78. Lo, A.W.I., et al., *DNA amplification by breakage/fusion/bridge cycles initiated by spontaneous telomere loss in a human cancer cell line.* Neoplasia, 2002. **4**(6): p. 531-538.

79. Baird, D.M., *Mechanisms of telomeric instability.* Cytogenet.Genome Res., 2008. **122**(3-4): p. 308-314.

80. Muraki, K., et al., *Mechanisms of telomere loss and their consequences for chromosome instability.* Front Oncol, 2012. **2**: p. 135.

81. Ricchetti, M., B. Dujon, and C. Fairhead, *Distance from the chromosome end determines the efficiency of double strand break repair in subtelomeres of haploid yeast.* J Mol Biol, 2003. **328**(4): p. 847-62.

82. Oikawa, S. and S. Kawanishi, *Site-specific DNA damage at GGG sequence by oxidative stress may accelerate telomere shortening.* Febs Letters, 1999. **453**(3): p. 365-368.

83. Proctor, C.J. and T.B.L. Kirkwood, *Modelling telomere shortening and the role of oxidative stress.* Mechanisms of Ageing and Development, 2002. **123**(4): p. 351-363.

84. von Zglinicki, T., R. Pilger, and N. Sitte, *Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts.* Free Radical Biology and Medicine, 2000. **28**(1): p. 64-74.

85. Greco, V., et al., *Histochemistry of the colonic epithelial mucins in normal subjects and in patients with ulcerative colitis. A qualitative and histophotometric investigation.* Gut, 1967. **8**(5): p. 491-6.

86. Myung, S.J., et al., *Clinical usefulness of telomerase for the detection of colon cancer in ulcerative colitis patients.* J.Gastroenterol.Hepatol., 2005. **20**(10): p. 1578-1583.

87. Risques, R.A., et al., *Ulcerative colitis is a disease of accelerated colon aging: evidence from telomere attrition and DNA damage.* Gastroenterology, 2008. **135**(2): p. 410-418.

88. O'Sullivan, J., et al., *Telomere length in the colon declines with age: a relation to colorectal cancer?* Cancer Epidemiol.Biomarkers Prev., 2006. **15**(3): p. 573-577.

89. Kinouchi, Y., et al., *Telomere shortening in the colonic mucosa of patients with ulcerative colitis.* J.Gastroenterol., 1998. **33**(3): p. 343-348.

90. O'Sullivan, J.N., et al., *Chromosomal instability in ulcerative colitis is related to telomere shortening.* Nat.Genet., 2002. **32**(2): p. 280-284.

91. di Fagagna, F.D., et al., *A DNA damage checkpoint response in telomere-initiated senescence.* Nature, 2003. **426**(6963): p. 194-198.

92. Hayashi, M.T., et al., *A telomere-dependent DNA damage checkpoint induced by prolonged mitotic arrest.* Nat Struct Mol Biol, 2012. **19**(4): p. 387-94.

93. Cosme-Blanco, W. and S. Chang, *Dual roles of telomere dysfunction in initiation and suppression of tumorigenesis.* Exp.Cell Res., 2008. **314**(9): p. 1973-1979.

94. Artandi, S.E. and L.D. Attardi, *Pathways connecting telomeres and p53 in senescence, apoptosis, and cancer.* Biochem.Biophys.Res.Commun., 2005. **331**(3): p. 881-890.

95. Artandi, S.E. and R.A. DePinho, *Telomeres and telomerase in cancer.* Carcinogenesis, 2010. **31**(1): p. 9-18.

96. Shay, J.W. and S. Bacchetti, *A survey of telomerase activity in human cancer.* Eur J Cancer, 1997. **33**(5): p. 787-91.

97. Chen, C.H. and R.J. Chen, *Prevalence of telomerase activity in human cancer.* J Formos Med Assoc, 2011. **110**(5): p. 275-89.

98. May, K.M. and K.G. Hardwick, *The spindle checkpoint.* J Cell Sci, 2006. **119**(Pt 20): p. 4139-42.

99. Musacchio, A. and E.D. Salmon, *The spindle-assembly checkpoint in space and time.* Nat Rev Mol Cell Biol, 2007. **8**(5): p. 379-93.

100. Fang, G., *Checkpoint protein BubR1 acts synergistically with Mad2 to inhibit anaphase-promoting complex.* Mol Biol Cell, 2002. **13**(3): p. 755-66.

101. Rieder, C.L. and H. Maiato, *Stuck in division or passing through: what happens when cells cannot satisfy the spindle assembly checkpoint.* Dev Cell, 2004. **7**(5): p. 637-51.

102. Weaver, B.A. and D.W. Cleveland, *Decoding the links between mitosis, cancer, and chemotherapy: The mitotic checkpoint, adaptation, and cell death.* Cancer Cell, 2005. **8**(1): p. 7-12.

103. Cahill, D.P., et al., *Mutations of mitotic checkpoint genes in human cancers.* Nature, 1998. **392**(6673): p. 300-3.

104. Jallepalli, P.V. and C. Lengauer, *Chromosome segregation and cancer: cutting through the mystery.* Nat Rev Cancer, 2001. **1**(2): p. 109-17.

105. Ricke, R.M. and J.M. van Deursen, *Aneuploidy in health, disease, and aging.* J Cell Biol, 2013. **201**(1): p. 11-21.

106. Ikawa-Yoshida, A., et al., *Contribution of BubR1 to oxidative stress-induced aneuploidy in p53-deficient cells.* Cancer Med, 2013. **2**(4): p. 447-56.

107. Ha, G.H. and E.K. Breuer, *Mitotic Kinases and p53 Signaling.* Biochem Res Int, 2012. **2012**: p. 195903.

108. Vader, G. and S.M. Lens, *The Aurora kinase family in cell division and cancer.* Biochim Biophys Acta, 2008. **1786**(1): p. 60-72.

109. Marumoto, T., D. Zhang, and H. Saya, *Aurora-A - a guardian of poles.* Nat Rev Cancer, 2005. **5**(1): p. 42-50.

110. Vogel, C., et al., *Crosstalk of the mitotic spindle assembly checkpoint with p53 to prevent polyploidy.* Oncogene, 2004. **23**(41): p. 6845-53.

111. Giet, R., C. Petretti, and C. Prigent, *Aurora kinases, aneuploidy and cancer, a coincidence or a real link?* Trends Cell Biol, 2005. **15**(5): p. 241-50.

112. Burum-Auensen, E., et al., *Spindle proteins Aurora A and BUB1B, but not Mad2, are aberrantly expressed in dysplastic mucosa of patients with longstanding ulcerative colitis.* J Clin Pathol, 2007. **60**(12): p. 1403-8.

113. Nikonova, A.S., et al., *Aurora A kinase (AURKA) in normal and pathological cell division.* Cell Mol Life Sci, 2013. **70**(4): p. 661-87.

114. Baba, Y., et al., *Aurora-A expression is independently associated with chromosomal instability in colorectal cancer.* Neoplasia, 2009. **11**(5): p. 418-25.

115. Anand, S., S. Penrhyn-Lowe, and A.R. Venkitaraman, *AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol.* Cancer Cell, 2003. **3**(1): p. 51-62.

116. Bischoff, J.R., et al., *A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers.* EMBO J, 1998. **17**(11): p. 3052-65.

117. De Angelis, P.M., et al., *Chromosomal gains and losses in primary colorectal carcinomas detected by CGH and their associations with tumour DNA ploidy, genotypes and phenotypes.* British Journal of Cancer, 1999. **80**(3-4): p. 526-535.

118. Barr, A.R. and F. Gergely, *Aurora-A: the maker and breaker of spindle poles.* J Cell Sci, 2007. **120**(Pt 17): p. 2987-96.

119. Zhang, D., et al., *Aurora A overexpression induces cellular senescence in mammary gland hyperplastic tumors developed in p53-deficient mice.* Oncogene, 2008. **27**(31): p. 4305-14.

120. Katayama, H., et al., *Phosphorylation by aurora kinase A induces Mdm2-mediated destabilization and inhibition of p53.* Nat Genet, 2004. **36**(1): p. 55-62.

121. Wu, C.C., et al., *p53 negatively regulates Aurora A via both transcriptional and posttranslational regulation.* Cell Cycle, 2012. **11**(18): p. 3433-3442.

122. Oren, M., W. Maltzman, and A.J. Levine, *Post-translational regulation of the 54K cellular tumor antigen in normal and transformed cells.* Mol Cell Biol, 1981. **1**(2): p. 101-10.

123. Giaccia, A.J. and M.B. Kastan, *The complexity of p53 modulation: emerging patterns from divergent signals.* Genes Dev, 1998. **12**(19): p. 2973-83.

124. Ashcroft, M. and K.H. Vousden, *Regulation of p53 stability.* Oncogene, 1999. **18**(53): p. 7637-43.

125. Soussi, T., *The history of p53. A perfect example of the drawbacks of scientific paradigms.* EMBO Rep, 2010. **11**(11): p. 822-6.

126. Levine, A.J. and M. Oren, *The first 30 years of p53: growing ever more complex.* Nat Rev Cancer, 2009. **9**(10): p. 749-58.

127. Vousden, K.H. and X. Lu, *Live or let die: the cell's response to p53.* Nat Rev Cancer, 2002. **2**(8): p. 594-604.

128. van Schaik, F.D., et al., *Role of immunohistochemical markers in predicting progression of dysplasia to advanced neoplasia in patients with ulcerative colitis.* Inflamm Bowel Dis, 2012. **18**(3): p. 480-8.

129. Meguerditchian, A.N. and K. Bullard Dunn, *Biomarkers and targeted therapeutics in colorectal cancer.* Surg Oncol Clin N Am, 2013. **22**(4): p. 841-55.

130. Nasierowska-Guttmejer, A., et al., *p53 protein accumulation and p53 gene mutation in colorectal cancer.* Pathol Oncol Res, 2000. **6**(4): p. 275-9.

131. Hammel, P., et al., *Correlations between p53-protein accumulation, serum antibodies and gene mutation in colorectal cancer.* Int J Cancer, 1999. **81**(5): p. 712-8.

132. Schjolberg, A.R., et al., *Aneuploidy Is Associated with TP53 Expression but not with BRCA1 or TERT Expression in Sporadic Colorectal Cancer.* Anticancer Research, 2009. **29**(11): p. 4381-4387.

133. Deangelis, P., et al., *P53 Expression Is Associated with a High-Degree of Tumor DNA Aneuploidy and Incidence of P53 Gene Mutation, and Is Localized to the Aneuploid Component in Colorectal Carcinomas.* International Journal of Oncology, 1993. **3**(2): p. 305-312.

134. Ghavam-Nasiri, M.R., et al., *Expression of p53 in colorectal carcinoma: correlation with clinicopathologic features.* Arch Iran Med, 2007. **10**(1): p. 38-42.

135. Russo, A., et al., *The TP53 colorectal cancer international collaborative study on the prognostic and predictive significance of p53 mutation: influence of tumor site, type of mutation, and adjuvant treatment.* J Clin Oncol, 2005. **23**(30): p. 7518-28.

136. Soong, R., et al., *Prognostic significance of TP53 gene mutation in 995 cases of colorectal carcinoma. Influence of tumour site, stage, adjuvant chemotherapy and type of mutation.* Eur J Cancer, 2000. **36**(16): p. 2053-60.

137. Chang, S.C., et al., *Genetic alteration of p53, but not overexpression of intratumoral p53 protein, or serum p53 antibody is a prognostic factor in sporadic colorectal adenocarcinoma.* Int J Oncol, 2005. **26**(1): p. 65-75.

138. Clausen, O.P., et al., *Association of p53 accumulation with TP53 mutations, loss of heterozygosity at 17p13, and DNA ploidy status in 273 colorectal carcinomas.* Diagn Mol Pathol, 1998. **7**(4): p. 215-23.

139. Harpaz, N., et al., *p53 protein expression in ulcerative colitis-associated colorectal dysplasia and carcinoma.* Hum Pathol, 1994. **25**(10): p. 1069-74.

140. Ilyas, M. and I.C. Talbot, *p53 expression in ulcerative colitis: a longitudinal study.* Gut, 1995. **37**(6): p. 802-4.

141. Leedham, S.J., et al., *Clonality, founder mutations, and field cancerization in human ulcerative colitis-associated neoplasia.* Gastroenterology, 2009. **136**(2): p. 542-50 e6.

142. Mattar, R., A.M. Alexandrino, and A.A. Laudanna, *Infrequent p53 gene alterations in ulcerative colitis.* Braz J Med Biol Res, 1999. **32**(9): p. 1083-8.

143. Burmer, G.C., et al., *Neoplastic progression in ulcerative colitis: histology, DNA content, and loss of a p53 allele.* Gastroenterology, 1992. **103**(5): p. 1602-10.

144. Brentnall, T.A., et al., *Mutations in the p53 gene: an early marker of neoplastic progression in ulcerative colitis.* Gastroenterology, 1994. **107**(2): p. 369-78.

145. Klump, B., et al., *Distribution of cell populations with DNA aneuploidy and p53 protein expression in ulcerative colitis.* Eur J Gastroenterol Hepatol, 1997. **9**(8): p. 789-94.

146. Wyatt, H.D., S.C. West, and T.L. Beattie, *InTERTpreting telomerase structure and function.* Nucleic Acids Res, 2010. **38**(17): p. 5609-22.

147. Cong, Y.S., W.E. Wright, and J.W. Shay, *Human telomerase and its regulation.* Microbiol Mol Biol Rev, 2002. **66**(3): p. 407-25, table of contents.

148. Collins, K. and J.R. Mitchell, *Telomerase in the human organism.* Oncogene, 2002. **21**(4): p. 564-79.

149. Greider, C.W. and E.H. Blackburn, *Identification of a specific telomere terminal transferase activity in Tetrahymena extracts.* Cell, 1985. **43**(2 Pt 1): p. 405-13.

150. Meyerson, M., *Role of telomerase in normal and cancer cells.* J Clin Oncol, 2000. **18**(13): p. 2626-34.

151. Cayuela, M.L., J.M. Flores, and M.A. Blasco, *The telomerase RNA component Terc is required for the tumour-promoting effects of Tert overexpression.* EMBO Rep, 2005. **6**(3): p. 268-74.

152. Bachor, C., O.A. Bachor, and P. Boukamp, *Telomerase is active in normal gastrointestinal mucosa and not up-regulated in precancerous lesions.* J.Cancer Res.Clin.Oncol., 1999. **125**(8-9): p. 453-460.

153. Martinez, P. and M.A. Blasco, *Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins.* Nat Rev Cancer, 2011. **11**(3): p. 161-76.

154. Hiyama, E., et al., *Immunohistochemical detection of telomerase (hTERT) protein in human cancer tissues and a subset of cells in normal tissues.* Neoplasia, 2001. **3**(1): p. 17-26.

155. Zheng, Y.L., et al., *Telomerase enzymatic component hTERT shortens long telomeres in human cells.* Cell Cycle, 2014. **13**(11).

156. Britt-Compton, B., et al., *Short telomeres are preferentially elongated by telomerase in human cells.* FEBS Lett., 2009. **583**(18): p. 3076-3080.

157. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.

158. Hackett, J.A. and C.W. Greider, *Balancing instability: dual roles for telomerase and telomere dysfunction in tumorigenesis.* Oncogene, 2002. **21**(4): p. 619-26.

159. Blasco, M.A., *Telomeres and human disease: ageing, cancer and beyond.* Nat.Rev.Genet., 2005. **6**(8): p. 611-622.

160. Baird, D.M., *Variation at the TERT locus and predisposition for cancer.* Expert.Rev.Mol.Med., 2010. **12**: p. e16.

161. Aust, D.E., et al., *Chromosomal alterations in ulcerative colitis-related and sporadic colorectal cancers by comparative genomic hybridization.* Hum Pathol, 2000. **31**(1): p. 109-14.

162. Chadeneau, C., et al., *Telomerase activity associated with acquisition of malignancy in human colorectal cancer.* Cancer Res, 1995. **55**(12): p. 2533-6.

163. Usselmann, B., et al., *Deficiency of colonic telomerase in ulcerative colitis.* Am J Gastroenterol, 2001. **96**(4): p. 1106-12.

164. Risques, R.A., et al., *Ulcerative colitis-associated colorectal cancer arises in a field of short telomeres, senescence, and inflammation.* Cancer Res., 2011. **71**(5): p. 1669-1679.

165. Kleideiter, E., et al., *Telomerase activity in chronic inflammatory bowel disease.* Dig Dis Sci, 2003. **48**(12): p. 2328-32.

166. Holzmann, K., et al., *Telomerase activity in long-standing ulcerative colitis.* Anticancer Res, 2000. **20**(5C): p. 3951-5.

167. Engelhardt, M., et al., *Telomerase and telomere length in the development and progression of premalignant lesions to colorectal cancer.* Clin Cancer Res, 1997. **3**(11): p. 1931-41.

168. Sipos, F., et al., *Elevated insulin-like growth factor 1 receptor, hepatocyte growth factor receptor and telomerase protein expression in mild ulcerative colitis.* Scand J Gastroenterol, 2008. **43**(3): p. 289-98.

169. Bronner, M.P., et al., *Genomic biomarkers to improve ulcerative colitis neoplasia surveillance.* Am J Pathol, 2008. **173**(6): p. 1853-60.

170. Salk, J.J., et al., *Clonal expansions in ulcerative colitis identify patients with neoplasia.* Proc.Natl.Acad.Sci.U.S.A, 2009. **106**(49): p. 20871-20876.

171. Haghighi, M.M., et al., *Telomere shortening: a biological marker of sporadic colorectal cancer with normal expression of p53 and mismatch repair proteins.* Genet Test Mol Biomarkers, 2014. **18**(4): p. 236-44.

172. Houben, J.M., et al., *Telomere length assessment: biomarker of chronic oxidative stress?* Free Radic Biol Med, 2008. **44**(3): p. 235-46.

173. Damjanovic, A.K., et al., *Accelerated telomere erosion is associated with a declining immune function of caregivers of Alzheimer's disease patients.* J Immunol, 2007. **179**(6): p. 4249-54.

174. Valdes, A.M., et al., *Obesity, cigarette smoking, and telomere length in women.* Lancet, 2005. **366**(9486): p. 662-4.

175. Morla, M., et al., *Telomere shortening in smokers with and without COPD.* European Respiratory Journal, 2006. **27**(3): p. 525-528.

176. Shalev, I., et al., *Exposure to violence during childhood is associated with telomere erosion from 5 to 10 years of age: a longitudinal study.* Molecular Psychiatry, 2013. **18**(5): p. 576-581.

177. Cunningham, J.M., et al., *Telomere length varies by DNA extraction method: implications for epidemiologic research.* Cancer Epidemiol Biomarkers Prev, 2013. **22**(11): p. 2047-54.

178. Vindelov, L.L., I.J. Christensen, and N.I. Nissen, *A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis.* Cytometry, 1983. **3**(5): p. 323-7.

179. Montpetit, A.J., et al., *Telomere length: a review of methods for measurement.* Nurs Res, 2014. **63**(4): p. 289-99.

180. Aubert, G., M. Hills, and P.M. Lansdorp, *Telomere length measurement-caveats and a critical assessment of the available technologies and tools.* Mutat Res, 2012. **730**(1-2): p. 59-67.

181. Vera, E. and M.A. Blasco, *Beyond average: potential for measurement of short telomeres.* Aging (Albany NY), 2012. **4**(6): p. 379-92.

182. Kimura, M., et al., *Offspring's leukocyte telomere length, paternal age, and telomere elongation in sperm.* PLoS Genet, 2008. **4**(2): p. e37.

183. Rufer, N., et al., *Telomere length dynamics in human lymphocyte subpopulations measured by flow cytometry.* Nat Biotechnol, 1998. **16**(8): p. 743-7.

184. Baerlocher, G.M., et al., *Flow cytometry and FISH to measure the average length of telomeres (flow FISH).* Nat.Protoc., 2006. **1**(5): p. 2365-2376.

185. Baerlocher, G.M., et al., *Telomere length measurement by fluorescence in situ hybridization and flow cytometry: tips and pitfalls.* Cytometry, 2002. **47**(2): p. 89-99.

186. Cawthon, R.M., *Telomere measurement by quantitative PCR.* Nucleic Acids Res., 2002. **30**(10): p. e47.

187. Ourliac-Garnier, I. and A. Londono-Vallejo, *Telomere length analysis by quantitative fluorescent in situ hybridization (Q-FISH).* Methods Mol Biol, 2011. **735**: p. 21-31.

188. Takubo, K., et al., *Changes of telomere length with aging.* Geriatr.Gerontol.Int., 2010. **10 Suppl 1**: p. S197-S206.

189. Ricciardi, M., M. Krampera, and M. Chilosi, *Quantitative fluorescence in situ hybridization on paraffin embedded tissue.* Methods Mol Biol, 2013. **976**: p. 167-73.

190. Meeker, A.K., et al., *Telomere length assessment in human archival tissues: combined telomere fluorescence in situ hybridization and immunostaining.* Am J Pathol, 2002. **160**(4): p. 1259-68.

191. Canela, A., et al., *High-throughput telomere length quantification by FISH and its application to human population studies.* Proc Natl Acad Sci U S A, 2007. **104**(13): p. 5300-5.

192. Baird, D.M., et al., *Extensive allelic variation and ultrashort telomeres in senescent human cells.* Nat.Genet., 2003. **33**(2): p. 203-207.

193. Bendix, L., et al., *The load of short telomeres, estimated by a new method, Universal STELA, correlates with number of senescent cells.* Aging Cell, 2010. **9**(3): p. 383-397.

194. VanGuilder, H.D., K.E. Vrana, and W.M. Freeman, *Twenty-five years of quantitative PCR for gene expression analysis.* Biotechniques, 2008. **44**(5): p. 619-26.

195. O'Callaghan, N.J. and M. Fenech, *A quantitative PCR method for measuring absolute telomere length.* Biol.Proced.Online., 2011. **13**: p. 3.

196. Cawthon, R.M., *Telomere length measurement by a novel monochrome multiplex quantitative PCR method.* Nucleic Acids Res., 2009. **37**(3): p. e21.

197. Aviv, A., et al., *Impartial comparative analysis of measurement of leukocyte telomere length/DNA content by Southern blots and qPCR.* Nucleic Acids Res, 2011. **39**(20): p. e134.

198. Bendix, L., et al., *Longitudinal Changes in Leukocyte Telomere Length and Mortality in Humans.* J Gerontol A Biol Sci Med Sci, 2013.

199. Zanet, D.L., et al., *Blood and dried blood spot telomere length measurement by qPCR: assay considerations.* PLoS One, 2013. **8**(2): p. e57787.

200. Burry, R.W., *Controls for immunocytochemistry: an update.* J Histochem Cytochem, 2011. **59**(1): p. 6-12.

201. Metz, B., et al., *Identification of formaldehyde-induced modifications in proteins: reactions with model peptides.* J Biol Chem, 2004. **279**(8): p. 6235-43.

202. Werner, M., et al., *Effect of formalin tissue fixation and processing on immunohistochemistry.* Am J Surg Pathol, 2000. **24**(7): p. 1016-9.

203. Hoos, A., et al., *Validation of tissue microarrays for immunohistochemical profiling of cancer specimens using the example of human fibroblastic tumors.* Am J Pathol, 2001. **158**(4): p. 1245-51.

204. Camp, R.L., L.A. Charette, and D.L. Rimm, *Validation of tissue microarray technology in breast carcinoma.* Lab Invest, 2000. **80**(12): p. 1943-9.

205. Wu, Y.L., et al., *Immunodetection of human telomerase reverse-transcriptase (hTERT) re-appraised: nucleolin and telomerase cross paths.* J Cell Sci, 2006. **119**(Pt 13): p. 2797-806.

206. Burum-Auensen, E., et al., *Subcellular localization of the spindle proteins Aurora A, Mad2, and BUBR1 assessed by immunohistochemistry.* J Histochem Cytochem, 2007. **55**(5): p. 477-86.

207. Nollau, P. and C. Wagener, *Methods for detection of point mutations: performance and quality assessment. IFCC Scientific Division, Committee on Molecular Biology Techniques.* Clin Chem, 1997. **43**(7): p. 1114-28.

208. Ekstrom, P.O., D.J. Warren, and W.G. Thilly, *Separation principles of cycling temperature capillary electrophoresis.* Electrophoresis, 2012. **33**(7): p. 1162-8.

209. Lishanski, A., E.A. Ostrander, and J. Rine, *Mutation detection by mismatch binding protein, MutS, in amplified DNA: application to the cystic fibrosis gene.* Proc Natl Acad Sci U S A, 1994. **91**(7): p. 2674-8.

210. Ferrari, M., P. Carrera, and L. Cremonesi, *Different approaches to molecular scanning of point mutations in genetic diseases.* Pure and Applied Chemistry, 1996. **68**(10): p. 1913-1918.

211. Khrapko, K., et al., *Constant denaturant capillary electrophoresis (CDCE): a high resolution approach to mutational analysis.* Nucleic Acids Res, 1994. **22**(3): p. 364-9.

212. Bjorheim, J., G. Gaudernack, and P.O. Ekstrom, *Mutation analysis of TP53 exons 5-8 by automated constant denaturant capillary electrophoresis.* Tumour Biol, 2001. **22**(5): p. 323-7.

213. Ekstrom, P.O., et al., *Analysis of mutational spectra by denaturing capillary electrophoresis.* Nat Protoc, 2008. **3**(7): p. 1153-66.

214. Itzkowitz, S.H., *Fingerprinting the colon?* Inflamm Bowel Dis, 2003. **9**(5): p. 338-9.

215. Løfberg, R., et al., *Flow cytometric DNA analysis in longstanding ulcerative colitis: a method of prediction of dysplasia and carcinoma development?* Gut, 1987. **28**(9): p. 1100-6.

216. Indran, I.R., M.P. Hande, and S. Pervaiz, *hTERT overexpression alleviates intracellular ROS production, improves mitochondrial function, and inhibits ROS-mediated apoptosis in cancer cells.* Cancer Res, 2011. **71**(1): p. 266-76.

217. Suram, A., et al., *Oncogene-induced telomere dysfunction enforces cellular senescence in human cancer precursor lesions.* EMBO J, 2012. **31**(13): p. 2839-51.

218. Cho, J.H. and S.R. Brant, *Recent insights into the genetics of inflammatory bowel disease.* Gastroenterology, 2011. **140**(6): p. 1704-12.

219. Yoshida, T., et al., *Diverse p53 alterations in ulcerative colitis-associated low-grade dysplasia: full-length gene sequencing in microdissected single crypts.* J Pathol, 2003. **199**(2): p. 166-75.

220. Lashner, B.A., et al., *Evaluation of the usefulness of testing for p53 mutations in colorectal cancer surveillance for ulcerative colitis.* Am J Gastroenterol, 1999. **94**(2): p. 456-62.

221. Levine, D.S., et al., *Distribution of aneuploid cell populations in ulcerative colitis with dysplasia or cancer.* Gastroenterology, 1991. **101**(5): p. 1198-210.

222. Willenbucher, R.F., et al., *Chromosomal alterations in ulcerative colitis-related neoplastic progression.* Gastroenterology, 1997. **113**(3): p. 791-801.

223. Salk, J.J., et al., *Clonal Expansions and Short Telomeres Are Associated with Neoplasia in Early-onset, but not Late-onset, Ulcerative Colitis.* Inflamm Bowel Dis, 2013. **19**(12): p. 2593-602.

224. Brackmann, S., et al., *Two distinct groups of colorectal cancer in inflammatory bowel disease.* Inflamm Bowel Dis, 2009. **15**(1): p. 9-16.

225. Kuo, L.J. and L.X. Yang, *Gamma-H2AX - a novel biomarker for DNA double-strand breaks.* In Vivo, 2008. **22**(3): p. 305-9.

226. Saeboe-Larssen, S., E. Fossberg, and G. Gaudernack, *Characterization of novel alternative splicing sites in human telomerase reverse transcriptase (hTERT): analysis of expression and mutual correlation in mRNA isoforms from normal and tumour tissues.* BMC Mol Biol, 2006. **7**: p. 26.

227. Suzuki, H., et al., *IGFBP7 is a p53-responsive gene specifically silenced in colorectal cancer with CpG island methylator phenotype.* Carcinogenesis, 2010. **31**(3): p. 342-349.

228. de Jesus, B.B. and M.A. Blasco, *Assessing cell and organ senescence biomarkers.* Circ Res, 2012. **111**(1): p. 97-109.

I

Friis-Ottessen et al. BMC Gastroenterology 2014, 14:8
http://www.biomedcentral.com/1471-230X/14/8

BMC Gastroenterology

RESEARCH ARTICLE Open Access

Telomere shortening correlates to dysplasia but not to DNA aneuploidy in longstanding ulcerative colitis

Mariann Friis-Ottessen[1,5*], Laila Bendix[2], Steen Kølvraa[2], Solveig Norheim-Andersen[3], Paula M De Angelis[1] and Ole Petter F Clausen[1,4]

Abstract

Background: Ulcerative colitis (UC) is a chronic, inflammatory bowel disease which may lead to dysplasia and adenocarcinoma in patients when long-lasting. Short telomeres have been reported in mucosal cells of UC patients. Telomeres are repetitive base sequences capping the ends of linear chromosomes, and protect them from erosion and subsequent wrongful recombination and end-to-end joining during cell division. Short telomeres are associated with the development of chromosomal instability and aneuploidy, the latter being risk factors for development of dysplasia and cancer. Specifically, the abrupt shortening of one or more telomeres to a critical length, rather than bulk shortening of telomeres, seems to be associated with chromosomal instability.

Methods: We investigated possible associations between dysplasia, aneuploidy and telomere status in a total of eight lesions from each of ten progressors and four nonprogressors suffering from longstanding UC. We have analyzed mean telomere length by qPCR, as well as the amount of ultra-short telomeres by the Universal STELA method.

Results: An increased amount of ultra-short telomeres, as well as general shortening of mean telomere length are significantly associated with dysplasia in longstanding UC. Furthermore, levels of ultra-short telomeres are also significantly increased in progressors (colons harbouring cancer/dysplasia and/or aneuploidy) compared to nonprogressors (without cancer/dysplasia/aneuploidy), whereas general shortening of telomeres did not show such associations.

Conclusions: Our data suggest that ultra-short telomeres may be more tightly linked to colorectal carcinogenesis through development of dysplasia in UC than general telomere shortening. Telomere status was not seen to associate with DNA aneuploidy.

Keywords: Ulcerative colitis, Ultra-short telomeres, Mean telomere length, DNA-aneuploidy, Dysplasia

Background

Telomeres are specialized structures capping all linear chromosomes, thereby protecting them from erosion during cell division and from wrongful recombination and end-to-end joining [1]. In cancer development telomeres have shown to play a dual role: when cell cycle checkpoints are intact they will act as a tumour suppressor mechanism by bringing about cellular senescence or apoptosis when the telomeres are too short for further division. If however, cell cycle checkpoints are disabled, cells are able to keep dividing past this point and possibly engage in development of chromosomal instability (CIN) [2,3]. It has previously been shown that human cancer cells may harbour ultra-short telomeres [4], and current beliefs are that it is not the shortening of the mean telomere length that might contribute to the development of CIN and altered morphology, but one or a few telomeres shortened to an "ultra-short" state [5,6] These ultra-short telomeres are suspected to form from an abrupt loss of a larger part of a telomere. Oxidative stress is known to cause single strand breaks in DNA

* Correspondence: mariann.friis-ottessen@rr-research.no
[1]Department of Pathology, Division of Diagnostics and Intervention, Oslo University Hospital, Rikshospitalet, Oslo, Norway
[5]OUS HF Rikshospitalet, Postboks 4950, Nydalen 0424, Oslo, Norway
Full list of author information is available at the end of the article

[7]. Breaks occurring within the telomeric region of a chromosome are often not repaired, thus leaving the cell with a shortened telomere after genome duplication [8]. Few options for measurement of the amount of ultra-short telomeres are available. Methods for detecting the shorter telomeres, like Q-FISH methods, are generally limited by the need for viable cells, or that they detect the percentage of cells containing short telomeres within a population. They are not able to detect a single, short telomere within a population [9,10]. In 2003, Baird and colleagues presented the single telomere length analysis, STELA, that is able to detect the shortest telomeres in a sample [11]. This analysis was originally limited to the XpYp telomeres, but has since been extended to several autosomal chromosomes as well [12]. This method detects chromosomes where the subtelomeric region is known, as it is dependent upon a chromosome-specific proximal primer. Bendix and colleagues then presented a method in 2010 that provides an estimate of the load of ultra-short telomeres in a DNA sample. This method is a version of the STELA, and was named the Universal-STELA, as it is not limited to chromosomes with known subtelomeric composition [13].

Ulcerative colitis (UC) is an inflammatory bowel disease characterized by inflammation-induced chronic destruction and regeneration of colonic mucosa, resulting in dysplasia and cancer development in some patients. Patients with long lasting UC are at increased risk for development of adenocarcinomas, usually following mucosal transition to dysplasia [14]. It is estimated that 10% of patients suffering from long-term UC will develop preneoplastic changes that might lead to cancer. These patients can be characterized as progressors, as they have preneoplastic changes in one or more locations that are associated with increased risk of progression to invasive cancer. A progressor is typically defined as a patient with dysplasia or cancer. We have also included detection of DNA-aneuploid cell populations as a trait of a progressor colon. A patient not presenting cancer, dysplasia or aneuploid cell populations will thus be considered a nonprogressor using these criteria.

Patients suffering from UC have been shown to harbour shorter telomeres in their colonic mucosa compared to age-matched controls [15-17]. UC-progressors harbour shorter telomeres than do UC-nonprogressors [18] and it has been shown that the mean telomere length of a UC-colon is mainly shortened during the first eight years following onset of the disease [17]. Differences in mean telomere length between low-grade dysplasia (LGD) and high-grade dysplasia (HGD) in the colonic mucosa of patients suffering from long-term UC, with the shortest telomeres reported from mucosa with LGD, have recently been reported [16]. DNA aneuploidy has also been associated with the development of malignancy in UC patients [19-21], and it has been shown that DNA aneuploidy is present in dysplastic as well as non-dysplastic areas of colonic mucosa [21-23]. Thus, both mucosal dysplasia [24,25] as well as DNA aneuploidy [21,26] might predict increased risk for progression to adenocarcinomas in the colon of patients with longstanding UC. Our aim was to examine telomere biology in preneoplastic lesions of patients with longstanding UC. Using the recently developed method, U-STELA, we investigated possible differences in the amount of ultra-short telomeres in aneuploid and diploid dysplastic and non-dysplastic lesions from UC-progressor colectomies. Nonprogressor UC-colons served as controls.

Methods

Histopathology

We examined colectomy specimens from 14 patients with longstanding UC from 10–30 years, resected at the Department of Surgery, Rikshospitalet, in the period 1985–1994. The colectomies are part of a patient material that has previously been investigated for aneuploidy-related parameters by our research group [27]. The material included 3 females and 11 males. All but 3 developed UC before the age of 50, and all patients presented extensive colitis.

Use of this material for research purposes has ethical approval from the Regional Ethical Committee, REK S-06062.

10 patients were classified as UC-progressors, i.e. presented with one or more areas of dysplasia or aneuploidy in their colonic mucosa. Tissue specimens for histopathological evaluation were harvested from eight consecutive locations throughout each bowel, in addition to samples collected from gross lesions if present. A total of 78 lesions were available for analysis and fixed in 70% ethanol and embedded in paraffin. Some of the specimens were fixed in formalin only. Tissue sections were cut at 4 μm thickness, stained with hematoxylin-eosin and evaluated independently by two experienced pathologists (OPFC, SNA) according to Riddell et al. [28]. The mucosal morphology was classified independently by each pathologist, and if the evaluation was the same, this diagnosis was used, if not, the lesions were viewed through a double microscope and discussed until consensus was reached. A portion of each fresh tissue specimen was used for isolation of single cells for DNA flow cytometry and for DNA extraction for molecular genetic analysis (see below). Biopsies from 4 colectomies classified as nonprogressor UC-colons were used as controls.

DNA-flow cytometry

Mucosa adjacent to that used for histopathological examination was mechanically disaggregated in PBS and EDTA using a scalpel. The resulting cell suspensions

were filtered through 70 μm nylon mesh and centrifuged at 1500 RPM for 5 minutes. The resulting pellet was resuspended in 1 ml PBS and fixed in 70% ice-cold Ethanol. Samples were stored overnight before preparation for DNA analysis using the procedure of Vindeløv [29], and were analyzed using a FACStar Plus cytometer equipped with 488 nm argon ion laser (BDIS, Spectra Physics, Mountain View, CA, USA). DNA content was assessed based on propidium iodide (PI) fluorescence emission. Aggregates and doublets were excluded from analyses using red fluorescence pulse width of the PI-signal. The purity of epithelial cells in the cell suspensions were analysed by bivariate forward scatter/PI fluorescence [30] and bivariate cytokeratin/PI fluorescence [31] and estimated to be >80%.

The amount of ultra-short telomeres measured by U-STELA

The Universal STELA method was performed as described by Bendix and colleagues [13], with a few minor alterations. Fifty ng DNA, extracted from each of the 78 lesions, was digested using a 1:1 mix of restriction enzymes NdeI and MseI. Ten ng of digested DNA was mixed with 50 μmol of the oligoes 42-mer and 11 + 2-mer. The mix was ramped from 65°C to 16°C over one hour, before adding 20U T4 ligase together with 1x NEB buffer 2 and 1x ATP (NEB). This mixture was left at 16°C for 12 hours before 20 U t4 ligase together with 10^{-3} μM telorette 3, 1x ATP and 1x NEB buffer 2 was added (to a total volume of 25 μl). The mixture was kept for 12 hours at 35°C, followed by a 20 minutes inactivating step at 65°C. PCR was then performed in a 12 μl reaction containing 40 pg template, 1x Failsafe PCR premix H (Epicentre), 0, 1 μM of each of primers Teltail and Adapter, and 1,25U Failsafe Enzyme mix (Epicentre). The reactions were performed on a 2720 Thermal Cycler (Applied Biosystems) with the conditions one cycle of 68°C for 5 minutes and 95°C for 2 minutes, followed by 24 cycles of 95°C for 15 seconds, 58°C for 20 seconds and 72°C for 11 minutes, and one cycle of 72°C for 15 minutes. The PCR-products were separated on a 0, 85% TBE Seakem agarose gel at 5 V/cm, and transferred to a positively charged nylon membrane by Southern blotting using a vacuum blotter. The blotted DNA was then hybridized overnight to a digoxigenin (DIG)-labelled probe specific for the telomeric sequence and further incubated with a DIG-specific antibody coupled to alkaline phosphatase (Roche). The probe was visualised by CDP-Star, a chemiluminescent substrate (Roche) and detected using a Kodak Image Station 4000R. The fragment sizes of the PCR-products are determined based on a DIG-labelled molecular weight marker (Roche), using Molecular Imaging from Carestream. A cut-off was set at 900 bp, as normal colonic mucosa had very few bands using this threshold (data not shown), and in UC-samples the bands were presented as single, separated bands. Bands were counted manually. An illustration of U-STELA results is shown in Figure 1.

Mean telomere length analysis

Telomere mean length quantification was carried out with an adaptation of the Q-PCR methods described by Cawthon [32,33]. TaqMan Copy Number Reference Assay RNase P (located at 14q11.2) or alternatively TaqMan Copy Number Reference Assay Tert (located on 5p5.33) (Both Applied Biosystems, Denmark) was run at recommended conditions to measure the single copy gene copy number (SCG). For measurement of telomere repeat copies (T) primers telg – 5′-ACA CTA AGG TTT GGG TTT GGG TTT GGG TTT GGG TTA GTG T-3′ and telc – 5′-TGT TAG GTA TCC CTA TCC CTA TCC CTA TCC CTA TCC CTA ACA-3′, [32] were added at a concentration of 0.5 μM to Ssofast EvaGreen Supermix with low ROX (BioRad, Denmark). Cycling conditions were: 2 min at 50°C, 2 min of 95°C, followed by 2 cycles of 95°C for 15 s, 52°C for 15 s and 36 cycles of 95°C for 15 s, 62°C for 15 s and 71°C for 15 s. The PCR was run on a 7900HT Fast Real-Time PCR System, as presented by Bendix and colleagues [34]. Following amplification, telomeric to single copy gene (T/SCG) ratios were derived from a standard curve using the 7900HT Sequence Detection System version SDS2.3 (Applied Biosystems, Denmark). The results were presented as T/SCG ratios. Samples were run twice in triplicates and the mean of these runs was used. Average coefficient of variance (CV) for T/SCG using RNAse P was 10.5%. Average CV for T/SCG using TERT was 11.3%.

We have used two different primer sets for SCG because the mean length is derived from the ratio between telomere product and single copy gene-product, and therefore there is a potential risk that we will either over- or underestimate the telomere length of the lesions comprised of aneuploid cells if the chosen SCG should be duplicated or deleted in these cells. TERT was chosen to supplement the RNAseP. TERT is located on chromosome 5p, a region often amplified in aneuploid, sporadic colorectal cancers [35] as well as in aneuploid UC [36]. By comparing the T/SCG-ratios derived from the aneuploid lesions alone, we note correlation in all but six lesions, four of which are adenocarcinomas (Additional file 1). To further overcome this potential limitation we repeated statistical analysis with only diploid lesions (presented in Additional file 2). Based on these results RNAse P was selected as SCG for our analyses.

Statistics

The relationships between mean telomere length/amounts of ultra short telomeres and morphological

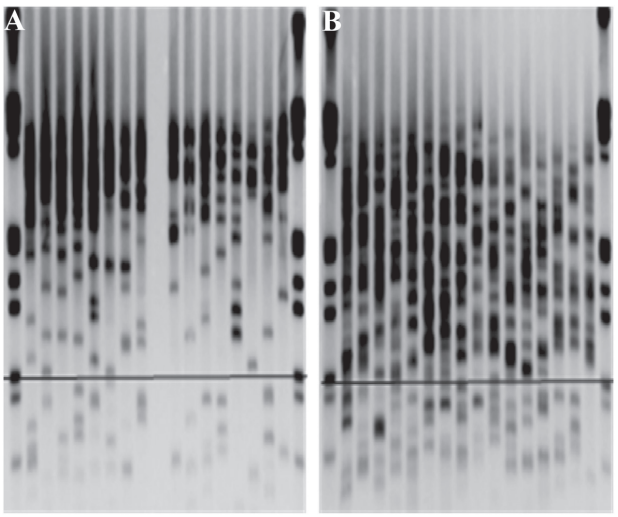


Figure 1 The Universal STELA. Excerpts of two typical membranes generated by a Southern blot, visualizing the ultra-short telomeres as analysed by the Universal STELA. Each lane represents a PCR-reaction. Eight separate PCR-reactions are performed for each lesion analysed. The horizontal bar marks 900 basepairs. Bands below this bar represent single telomeres and are counted for the analyses. **A)** Two lesions from a nonprogressor. **B)** Two lesions from a progressor.

changes/DNA-ploidy-status were evaluated using non-parametric t-tests and one-way ANOVA and the multivariable, general linear model (GLM). To address the possible differences generated by natural variation between patients we also utilized a mixed model analysis that took into account that each patient generated multiple lesions. Tests were performed in PASW statistics 18 (Chicago, Illinois) and in GraphPad Prism (San Diego, California). All tests were two-sided and a p-level of 0.05 denoted significance.

P-values from t-tests, including the Universal-STELA and both SCG primers tested for measuring mean telomere length are listed in Additional file 2.

Results

Histopathology

From the analyzed 14 colectomies 10 colons revealed at least one area with dysplasia, adenocarcinoma or DNA aneuploidy out of the eight areas analyzed (see Table 1). Within the same lesion the mucosal morphology might differ; in such cases the highest degree of abnormality was registered. Of the 78 areas analyzed, 40 areas did not show dysplasia (51.3%), 15 areas were considered indefinite for dysplasia (19.2%), 17 areas had either low or high grade dysplasia (21.8%) and 6 adenocarcinomas (7.7%) were found. These ten colectomy specimens were therefore considered progressors, including one bowel with 4 areas indefinite for dysplasia and four non-dysplastic areas. Two lesions were not available for analyses. Four colectomies presented only diploid, non-dysplastic lesions. These were classified as nonprogressors and used as controls.

Due to limited amounts of DNA from some colectomy-samples only one of the methods could be performed on some of the cases (see Table 2).

DNA flow cytometry

The DNA ploidy status of the colectomies has been previously assessed [27]. Within the 10 progressor colons analyzed 57 lesions were diploid (70%) and 21 (30%) lesions harboured aneuploid populations (see Table 1). In addition one diploid and five aneuploid adenocarcinomas were recorded. Two lesions were not available for analyses. We detected both aneuploid and diploid non-dysplastic areas as well as aneuploid and diploid dysplastic areas. One colon was diploid all through, but presented multiple sites of dysplasia. All nonprogressor lesions were diploid.

The proportion of aneuploid cells varied from around 5% to 10% within the lesions presenting aneuploid cell populations, with exception of one case (S169) presenting aneuploid cell counts estimated to be between 15 and 50% and one cancer (S99) represented only in qPCR.

Ultra-short telomeres and associations

Overall we found a significant difference in the amount of ultra short telomeres between progressor and nonprogressor

Table 1 UC-patient materials

Sample ID	Aneuploid areas	Dysplasia				Adeno-carcinoma	Universal STELA	qPCR
		No	Indefinite	Low-grade	High-grade			
Nonprogressors								
S9	0	8	0	0	0	0	X	X
S48	0	8	0	0	0	0	X	X
S62	0	8	0	0	0	0	X	X
S216	0	7	0	0	0	0	X	
Total		**31**						
Progressors								
S30	1	7	0	0	0	1	X	
S70	0	6	0	0	1	1	X	X
S99	2	0	2	2	3	1		X
S132	3	1	5	2	0	0	X	X
S159	3	2	0	3	1	0		X
S169	6	1	1	2	1	3	X	X
S191	3	6	1	1	0	0		X
S199	1	5	1	1	0	0	X	X
C1514	2	7	1	0	0	0	X	X
C1729	0	4	4	0	0	0	X	X
Total	**21**	**39**	**15**	**11**	**6**	**6**		

colons ($p < 0.001$) (Figure 2A). This significance holds also when comparisons are restricted to diploid, non-dysplastic lesions of the progressors (see Additional file 2).

Within the progressors we defined four morphological parameters; non-dysplasia, indefinite for dysplasia, dysplasia and adenocarcinoma. Dysplasia comprises both high-grade and low-grade dysplasia, due to few dysplastic lesions and no statistical significant difference in the amount of ultra short telomeres detected between the two groups ($p = 0.534$).

Dividing the dysplastic lesions as mentioned previously, we noted a significant increase in the amount of ultra-short telomeres in areas indefinite for dysplasia when compared to non-dysplastic lesions ($p = 0.003$).

Table 2 Lesions available for telomere analyses

	Aneuploid lesions		Diploid lesions	
	U-STELA	qPCR	U-STELA	qPCR
Nonprogressor lesions				
Non-dysplasia	0	0	27	22
Dysplasia	0	0	0	0
Total	0	0	27	22
Progressor lesions				
Non-dysplasia	4	7	24	25
Dysplasia	8	13	15	24
Total	12	20	39	49

The amount of ultra short telomeres detected in lesions indefinite for dysplasia did not differ significantly from amounts detected in dysplastic lesions or to the adenocarcinomas (ANOVA with Bonferroni post hoc test) (Figure 2A). No significant difference in the levels of ultra short telomeres was detected between lesions with and without aneuploid populations. Within the diploid lesions of the progressors we found significantly raised levels of ultra short telomeres in the dysplastic lesions compared to the non-dysplastic lesions ($p = 0.014$), whereas no significant difference was detected between the aneuploid dysplastic areas compared to aneuploid non-dysplastic areas, and diploid dysplasia did not differ from aneuploid dysplasia.

Significant differences related to the location within the colon were not observed. Within the progressor colons we detected a trend towards elevated number of ultra short telomeres in the dysplastic lesions (indefinite for dysplasia and/or dysplasia) compared to the non-dysplastic lesions of the same colon. No such trend was detected when comparing diploid and aneuploid lesions with respect to the amount of ultra short telomeres, within a colon.

To further test if the amount of ultra short telomeres can be an independent predictor of dysplasia in progressor colons we built a model that included morphology, DNA ploidy status, duration of disease combined with onset of UC and location of the biopsy within the colon.

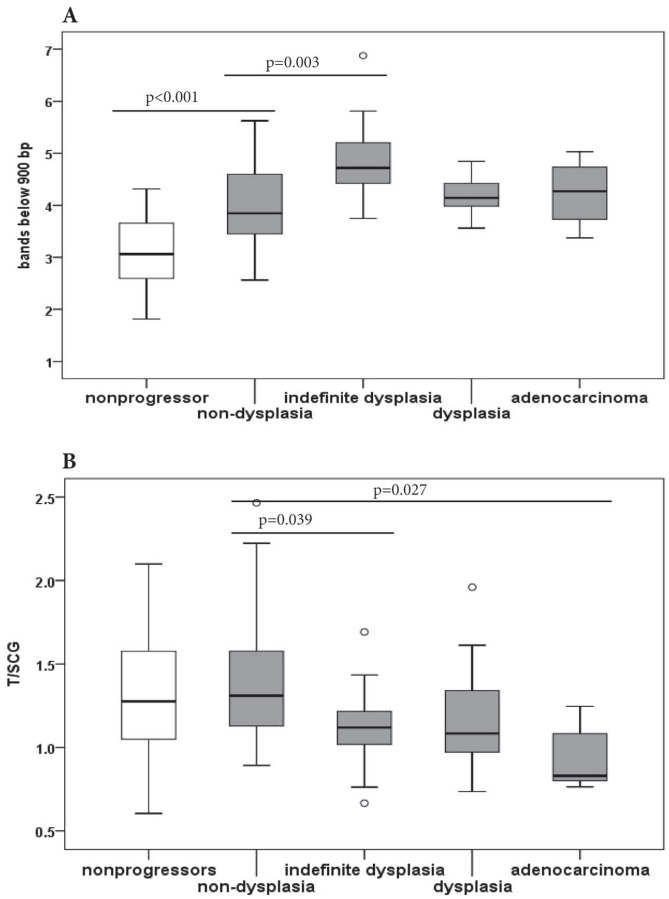


Figure 2 Ultra-short telomeres and mean telomere length in nonprogressors and progressors. A) The amount of ultra short telomeres differs significantly from nonprogressors to progressors ($p < 0.001$). Within progressors there is a significant rise in the amount of ultra short telomeres in lesions indefinite for dysplasia compared to non-dysplastic lesions. No difference is seen within the different degrees of dysplasia and adenocarcinoma. **B)** No significant difference was detected in mean telomere length from nonprogressors to progressors. Within the progressors we noted a significant shortening in mean telomere length in lesions indefinite for dysplasia and in adenocarcinomas from non-dysplastic lesions. (ANOVA with Bonferroni post hoc test).

This was tested using a multivariable, general linear model (multivariable GLM). The results of this test supported our findings of ultra short telomeres being a possible predictor of dysplasia in progressors ($p = 0.007$) even when the other parameters are taken into account.

As all colectomies contributed eight lesions to our analyses we also performed a mixed model analysis that considers the natural variation between each patient. Still we found a significant difference between progressor and nonprogressor cases ($p = 0.014$). Within the ten progressor colectomies we found a significant difference between the different degrees of dysplasia when LGD, HGD and adenocarcinomas were combined ($p = 0.033$). No significant difference was seen comparing diploid and aneuploid lesions.

Other parameters tested were patients' age at onset of disease, and duration of disease, as our patient material varied greatly with respect to both parameters. No association between the amount of ultra-short telomeres and either of these parameters was seen (data not shown). Gender did not associate with the amount of ultra-short telomeres.

Mean telomere length and associations

In this analysis we did not find any difference in mean telomere length between progressors and nonprogressors, neither in the whole material, nor when analysing only non-dysplastic, diploid lesions (Figure 2B and Additional file 2).

Within the progressors a significant shortening in mean telomere length was seen when comparing a group comprising lesions indefinite for dysplasia and dysplasia, to non-dysplastic lesions of the progressors-colons ($p = 0.002$) (see Additional file 2).

This remained significant also when restricting the analysis to diploid lesions alone ($p = 0.008$). No significant difference in mean telomere length was found comparing indefinite for dysplasia, dysplasia and adenocarcinoma, but we noted a significant reduction in mean telomere length for lesions indefinite for dysplasia as well as for adenocarcinomas compared to the non-dysplastic lesions of the progressors (ANOVA $p = 0.003$; Bonferroni post hoc test, $p = 0.039$ and $p = 0.027$, respectively) (Figure 2B).

Between the segments of the colectomies we found within the progressors a significant reduction of mean telomere length in the transverse colon compared to the ascending colon (ANOVA, Bonferroni post hoc test, $p = 0.011$). The descending part of the colectomies did not differ from either the transverse or the ascending colon.

No significant difference was detected between the non-dysplastic and dysplastic aneuploid lesions, or between diploid and aneuploid dysplasia. When comparing different lesions within each progressor colon we detected a trend towards reduced mean telomere length in dysplastic versus non-dysplastic lesions within colons. No such trend was seen between diploid and aneuploid lesions of the colons.

We repeated the calculations with the mean length normalized to TERT. The findings were overall similar to those registered when normalizing to RNaseP, but difference between progressors/nonprogressors was now borderline significant ($p = 0.08$), and the difference in mean telomere length between diploid and aneuploid lesions within the progressors was statistically significant ($p = 0.04$) (see Additional file 2).

In a multivariable GLM where we tested for the effect of biopsy location, degree of dysplasia, DNA ploidy status and age at onset combined with duration of UC, we found that in the progressor colectomies, both location of the biopsy ($p = 0.006$) and degree of dysplasia ($p = 0.012$) might be individually influential to the mean telomere length.

Since each colectomy provided several lesions we performed a GLM considering the patient variations. No significant difference was found when comparing progressor colectomies to nonprogressor colectomies. Within the progressor cases there was a significantly longer mean telomere length in non-dysplastic compared to dysplastic lesions when dysplastic lesions contained all dysplasia (indefinite, LGD, HGD and adenocarcinoma) ($p = 0.028$). The significance disappeared when lesions indefinite for dysplasia was viewed as a separate group.

Mean telomere length did not associate with gender, patients' age at disease onset or to duration of UC.

Discussion

It has been suggested that it is not necessarily the general shortening of telomeres that causes chromosomal instability and aneuploidy, but rather one or more critically short telomeres [5]. We have examined the amount of ultra-short telomeres [13] in the colonic mucosal cells of patients suffering from long standing UC, who have developed dysplasia and/or DNA-aneuploidy. We compared these findings with those from patients suffering from long standing UC without displaying dysplasia or DNA-aneuploidy. Patients with long standing UC have previously been reported to exhibit shortening of mean telomere length in their colonic mucosa, but the amount of ultra-short telomeres has so far not been investigated.

One major finding is a highly significant increase in number of ultra-short telomeres in progressor colons compared to nonprogressor colons, both when including all lesions of the progressors as well as when restricting the progressor lesions to non-dysplastic, diploid lesions only. Furthermore, both simple pair-wise comparisons and multivariate analysis showed that the degree of increase in ultra-short telomeres associated much better with degree of dysplasia than with ploidy status, disease duration or location. This was seen whether we included the adenocarcinomas in the dysplasias, or not. In contrast; there was no difference in mean telomere length between nonprogressors and progressors, although we also here found an association of mean telomere length with dysplasia. The fact that the number of ultra-short telomeres was significantly higher in dysplastic areas compared to non-dysplastic areas suggests that the occurrence of ultra-short telomeres is an early event in the progression towards dysplasia and invasive cancer. This is further supported by the fact that the lesions defined as indefinite for dysplasia, which applies to epithelial changes that exceed the limit for regeneration but are insufficient for an unequivocal diagnosis of dysplasia [28], have significantly more ultra-short telomeres than non-dysplastic mucosa. Elevated numbers of ultra-short telomeres in dysplastic compared to nondysplastic areas were also seen comparing dysplastic to the non-dysplastic lesions using a mixed model analysis. The mixed model analysis compensates for inter-patient differences, and gives a strong indication that the differences seen are indeed caused by differences in the mucosal morphology.

Differences in the number of ultra-short telomeres were not seen between diploid and aneuploid lesions within the examined colectomies, when examined by the mixed model analysis.

Thus; increased numbers of ultra-short telomeres may be a marker of UC-colons harbouring dysplasia, cancer and/or aneuploidy, but may also just predict the future development of these conditions.

Short telomeres predispose to end-to-end fusions of chromatids, ultimately leading to chromosome breaks and chromosome losses [18,37]. We did not find any associations between the amount of ultra-short telomeres and aneuploidy. However; it has been previously shown, and confirmed by our results, that not all dysplasias are aneuploid. Aneuploidy develops in non-dysplastic as well as dysplastic mucosa [19,23], and associations between dysplasia and ploidy status respectively, to telomere shortening, may be different. We did not detect any statistically significant difference when comparing aneuploid progressor lesions to diploid progressor lesions, and diploid dysplasia did not differ from aneuploid dysplasia, further indicating that DNA aneuploidy is not strongly associated with elevated levels of ultra-short telomeres. However, we have analysed areas of UC-colons that had developed DNA-aneuploidy, and most lesions included a rather low amount of aneuploid cells (see Result section; DNA flow cytometry for details). We can therefore not exclude an association between aneuploid cells and ultra-short telomeres. To investigate this problem, pure populations of sorted aneuploid cells should be analyzed.

We find that dysplastic lesions in longstanding UC have significantly shorter mean telomere length compared to non-dysplastic areas, when we combine lesions indefinite for dysplasia and dysplastic lesions. This holds true also when separating lesions indefinite for dysplasia from dysplastic lesions: both parameters had significantly reduced mean telomere length compared to non-dysplastic lesions, but no difference was seen between lesions indefinite for dysplasia and the dysplastic lesions. This was seen whether we included adenocarcinomas in the dysplastic lesions or excluded them from the data completely, but not when adenocarcinoma was also included in the statistics as a separate group. The adenocarcinomas presented significantly shorter mean telomere length than seen in non-dysplastic lesions, whereas both indefinite for dysplasia and dysplastic lesion had no statistical significant difference in mean telomere length from non-dysplastic lesions. These results indicate a gradual shortening from areas indefinite for dysplasia through dysplasia to adenocarcinoma. Considered together with the results from the amount of ultra-short telomeres discussed above, our results seem to illustrate damage to telomeres by oxidative stress in the colonic mucosa of UC-patients, as oxidative stress may cause shortening of mean telomere length as well as an abrupt shortening of a single or of a few telomeres.

Shortening of mean telomere length in non-dysplastic mucosa of UC patients has previously been shown, and was also reported to be associated with chromosomal instability [18]. Shortened mean telomere length of the colonic mucosa has also been reported in association with development of colorectal cancer in patients suffering from UC [16,17]. Other parameters that have been found to associate with such development are morphological changes and development of DNA-aneuploidy [16,17,19,21,26,37,38]. Furthermore, it has been reported that patients suffering from UC have generally shorter mean telomere length in their colonic mucosa compared to non-UC control patients. The location of the biopsies within the bowel did not affect these results, and there are indications that the main shortening of telomeric length occurred during the initial eight years of disease [15-18]. It has also been shown by others that progressors have generally shorter telomeres than registered in nonprogressors [18]. The patients in the present study have suffered from UC from a minimum of 10 years, some as long as 30 years, and the mean telomere length in their colonic mucosa may therefore be reduced too much to detect possible differences between nonprogressors and progressors. This may be an explanation to the finding of no significant difference in mean telomere length between progressors and nonprogressors in our study. Another possible explanation might be that we have a rather small number of patients coupled with a rather high coefficient of variation for mean telomere length measurement with either of the single control genes used (10.5% and 11.3%).

In a recent report it was shown that patients developing UC after the age of 50 present longer telomeres than patients developing UC at an earlier age [39]. In our material only two progressor cases and one nonprogressor case had reached the age of 50 before reporting signs of UC, and none of them differed from the rest of the progressor cases with respect to mean telomere length or to the amount of ultra-short telomeres. A possible explanation for the lack of differences detected between these groups might also be severity of disease, as all patients included in our study had developed pancolitis before colectomy.

The difference we noted in the two SCGs used to estimate mean telomere length might be caused by amplification of chromosome arm 5p, the arm that contains the h-TERT locus. Amplification of 5p is often seen both in aneuploid, sporadic colorectal cancers [35] and even more frequently in aneuploid UC [36]. Duplication of h-TERT in some of the aneuploid lesions can report a too low T/SCG value in these samples, and lower the mean value of the combined

aneuploid populations. This will again produce a bigger difference in mean telomere length between the diploid and aneuploid populations, than what is actually the case, and ultimately lead to a false statistical significance. We therefore considered RNaseP to be a more accurate SCG for this analysis.

Expression of hTERT is considered the limiting factor of telomerase assembly, and thus of telomerase activity. The presence of hTERT elevation in UC with mild inflammation [40] could perhaps be a protective agent against development of ultra-short telomeres within nonprogressors.

An important finding in the present communication is that the telomere related abnormalities in mucosa from patients with long-standing UC are much less significant when measuring mean telomere length than when measuring number of ultra-short telomeres. This finding suggests that the mechanism behind telomere pathology is not only increased replication due to cell renewal, since this would have resulted in a decrease in mean telomere length. In contrast it has been shown in a model system [41], that stochastic telomere breaks resulting in an increase in number of ultra-short telomeres without concomitant decrease in mean telomere length can be the result of severe oxidative damage to telomeres. Our findings could suggest that oxidative damage is a major reason for telomere pathology in UC mucosa, a hypothesis that is supported by the finding of elevated levels of oxidative stress in the mucosa of UC patients [42,43].

To what extend the high frequency of ultra-short telomeres in colon mucosa cells of patients with UC have causal relationship with increased risk of UC-related colon cancer in these patients is still unknown. A carcinogenic pathway from short telomeres linked to breakage-fusion-bridge cycles to development of chromosome aberrations and aneuploidy, and ultimately to malignant transformation, has been indicated previously in a model system [44].

Conclusion

We have confirmed that mean telomere length is reduced in dysplastic colonic mucosa in progressor colons of longstanding UC compared to non-dysplastic mucosa, and shown that the amount of ultra-short telomeres is increased in the same situation. Furthermore, we have shown that the amount of ultra-short telomeres is significantly increased in colonic mucosa of progressors compared to nonprogressors, whereas such a difference was not found with respect to mean telomere length. This suggests that ultra-short telomeres are a more sensitive marker of tumour progression than mean telomere length in longstanding UC, and also that the Universal-STELA may be a more accurate estimator of a progressor than qPCR and mean telomere length measurement.

Additional files

Additional file 1: Mean telomere length estimated with two different single copy genes (SCG). The relation between mean telomere estimated with the regular SCG RNAseP (TS_RNAseP) and the mean telomere length with the alternative TERT gene (TS_TERT). There is correlation in all samples but six (marked with arrows). These six samples are from progressors and all are aneuploid. Of the six lesions four were adenocarcinomas, one was dysplastic and one was a non-dysplastic lesion.

Additional file 2: Mean and p-values from t-tests. Mean values and p-values of t-tests comparing progressors to nonprogressors, and comparing the different parameters from the progressor colons. Results are reported for U-STELA and for two different mean telomere analyses. One was using RNAseP as SCG, the other using TERT.

Competing interests

This work was supported by the South-Eastern Norway Regional Health Authority and by Stiftelsen UNI. These organisations had no role in the collection, analyses or interpretations of the data or in writing the report.

Authors' contributions

MFO performed the STELA-analyses, organized the databases, did the statistical analyses and wrote the paper. LB performed the qPCR-analyses and provided critical insights for the writing of the paper. PMD was involved in planning and organizing the project. OPC is the principal investigator, supervisor and guarantor of the study. SNA provided the patient material for the study. SK and the other authors were all involved in manuscript preparation, evaluation, editing and revision. All authors read and approved the final manuscript.

Author details

[1]Department of Pathology, Division of Diagnostics and Intervention, Oslo University Hospital, Rikshospitalet, Oslo, Norway. [2]Danish Aging Research Center, University of Southern Denmark, Vejle, Denmark. [3]Department of Pathology, Akershus University Hospital, Division of Medicine and Laboratory Sciences, University of Oslo, Oslo, Norway. [4]Department of Pathology, University of Oslo, Oslo, Norway. [5]OUS HF Rikshospitalet, Postboks 4950, Nydalen 0424 Oslo, Norway.

Received: 5 April 2013 Accepted: 30 December 2013
Published: 9 January 2014

References

1. Blackburn EH: **Switching and signaling at the telomere.** *Cell* 2001, **106**(6):661–673.
2. Wright WE, Shay JW: **The two-stage mechanism controlling cellular senescence and immortalization.** *Exp Gerontol* 1992, **27**(4):383–389.
3. Shay JW, Wright WE: **Telomeres and telomerase in normal and cancer stem cells.** *FEBS Lett* 2010, **584**(17):3819–3825.
4. Xu L, Blackburn EH: **Human cancer cells harbor T-stumps, a distinct class of extremely short telomeres.** *MolCell* 2007, **28**(2):315–327.
5. Hemann MT, Strong MA, Hao L-Y, Greider CW: **The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability.** *Cell* 2001, **107**(1):67–77.
6. Capper R, Britt-Compton B, Tankimanova M, Rowson J, Letsolo B, Man S, Haughton M, Baird DM: **The nature of telomere fusion and a definition of the critical telomere length in human cells.** *Genes Dev* 2007, **21**(19):2495–2508.
7. Vonzglinicki T, Saretzki G, Docke W, Lotze C: **Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts - a model for senescence.** *Exp Cell Res* 1995, **220**(1):186–193.
8. Richter T, Saretzki G, Nelson G, Melcher M, Olijslagers S, von Zglinicki T: **TRF2 overexpression diminishes repair of telomeric single-strand breaks and accelerates telomere shortening in human fibroblasts.** *Mech Ageing Dev* 2007, **128**(4):340–345.
9. O'Sullivan JN, Finley JC, Risques RA, Shen WT, Gollahon KA, Moskovitz AH, Gryaznov S, Harley CB, Rabinovitch PS: **Telomere length assessment in tissue sections by quantitative FISH: image analysis algorithms.** *Cytometry A* 2004, **58**(2):120–131.

10. Canela A, Vera E, Klatt P, Blasco MA: **High-throughput telomere length quantification by FISH and its application to human population studies.** *Proc Natl Acad Sci U S A* 2007, **104**(13):5300–5305.
11. Baird DM, Rowson J, Wynford-Thomas D, Kipling D: **Extensive allelic variation and ultrashort telomeres in senescent human cells.** *NatGenet* 2003, **33**(2):203–207.
12. Britt-Compton B, Rowson J, Locke M, Mackenzie I, Kipling D, Baird DM: **Structural stability and chromosome-specific telomere length is governed by cis-acting determinants in humans.** *Hum Mol Genet* 2006, **15**(5):725–733.
13. Bendix L, Horn PB, Jensen UB, Rubelj I, Kolvraa S: **The load of short telomeres, estimated by a new method, Universal STELA, correlates with number of senescent cells.** *Aging Cell* 2010, **9**(3):383–397.
14. Macdougall IP: **The cancer risk in ulcerative colitis.** *Lancet* 1964, **2**(7361):655–658.
15. Kinouchi Y, Hiwatashi N, Chida M, Nagashima F, Takagi S, Maekawa H, Toyota T: **Telomere shortening in the colonic mucosa of patients with ulcerative colitis.** *JGastroenterol* 1998, **33**(3):343–348.
16. Risques RA, Lai LA, Himmetoglu C, Ebaee A, Li L, Feng Z, Bronner MP, Al-Lahham B, Kowdley KV, Lindor KD, *et al*: **Ulcerative colitis-associated colorectal cancer arises in a field of short telomeres, senescence, and inflammation.** *Cancer Res* 2011, **71**(5):1669–1679.
17. Risques RA, Lai LA, Brentnall TA, Li L, Feng Z, Gallaher J, Mandelson MT, Potter JD, Bronner MP, Rabinovitch PS: **Ulcerative colitis is a disease of accelerated colon aging: evidence from telomere attrition and DNA damage.** *Gastroenterology* 2008, **135**(2):410–418.
18. O'Sullivan JN, Bronner MP, Brentnall TA, Finley JC, Shen WT, Emerson S, Emond MJ, Gollahon KA, Moskovitz AH, Crispin DA, *et al*: **Chromosomal instability in ulcerative colitis is related to telomere shortening.** *NatGenet* 2002, **32**(2):280–284.
19. Hammarberg C, Slezak P, Tribukait B: **Early detection of malignancy in ulcerative colitis. A flow-cytometric DNA study.** *Cancer* 1984, **53**(2):291–295.
20. Løfberg R, Tribukait B, Ost A, Brostrom O, Reichard H: **Flow cytometric DNA analysis in longstanding ulcerative colitis: a method of prediction of dysplasia and carcinoma development?** *Gut* 1987, **28**(9):1100–1106.
21. Levine DS, Rabinovitch PS, Haggitt RC, Blount PL, Dean PJ, Rubin CE, Reid BJ: **Distribution of aneuploid cell populations in ulcerative colitis with dysplasia or cancer.** *Gastroenterology* 1991, **101**(5):1198–1210.
22. Fozard JB, Quirke P, Dixon MF, Giles GR, Bird CC: **DNA aneuploidy in ulcerative colitis.** *Gut* 1986, **27**(12):1414–1418.
23. Meling GI, Clausen OP, Bergan A, Schjolberg A, Rognum TO: **Flow cytometric DNA ploidy pattern in dysplastic mucosa, and in primary and metastatic carcinomas in patients with longstanding ulcerative colitis.** *Br J Cancer* 1991, **64**(2):339–344.
24. Morson BC, Pang LS: **Rectal biopsy as an aid to cancer control in ulcerative colitis.** *Gut* 1967, **8**(5):423–434.
25. Riddell RH, Morson BC: **Value of sigmoidoscopy and biopsy in detection of carcinoma and premalignant change in ulcerative colitis.** *Gut* 1979, **20**(7):575–580.
26. Rubin CE, Haggitt RC, Burmer GC, Brentnall TA, Stevens AC, Levine DS, Dean PJ, Kimmey M, Perera DR, Rabinovitch PS: **DNA aneuploidy in colonic biopsies predicts future development of dysplasia in ulcerative colitis.** *Gastroenterology* 1992, **103**(5):1611–1620.
27. Burum-Auensen E, De Angelis PM, Schjolberg AR, Roislien J, Andersen SN, Clausen OP: **Spindle proteins Aurora A and BUB1B, but not Mad2, are aberrantly expressed in dysplastic mucosa of patients with longstanding ulcerative colitis.** *J Clin Pathol* 2007, **60**(12):1403–1408.
28. Riddell RH, Goldman H, Ransohoff DF, Appelman HD, Fenoglio CM, Haggitt RC, Ahren C, Correa P, Hamilton SR, Morson BC, *et al*: **Dysplasia in inflammatory bowel disease: standardized classification with provisional clinical applications.** *Hum Pathol* 1983, **14**(11):931–968.
29. Vindelov LL, Christensen IJ, Nissen NI: **A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis.** *Cytometry* 1983, **3**(5):323–327.
30. Geido E, Sciutto A, Rubagotti A, Oliani C, Monaco R, Risio M, Giaretti W: **Combined DNA flow cytometry and sorting with k-ras2 mutation spectrum analysis and the prognosis of human sporadic colorectal cancer.** *Cytometry* 2002, **50**(4):216–224.
31. Clausen OP, Andersen SN, Stroomkjaer H, Nielsen V, Rognum TO, Bolund L, Koolvraa S: **A strategy combining flow sorting and comparative genomic hybridization for studying genetic aberrations at different stages of colorectal tumorigenesis in ulcerative colitis.** *Cytometry* 2001, **43**(1):46–54.
32. Cawthon RM: **Telomere length measurement by a novel monochrome multiplex quantitative PCR method.** *Nucleic acids research* 2009, **37**(3):e21.
33. Cawthon RM: **Telomere measurement by quantitative PCR.** *Nucleic acids research* 2002, **30**(10):e47.
34. Bendix L, Thinggaard M, Fenger M, Kolvraa S, Avlund K, Linneberg A, Osler M: **Longitudinal changes in leukocyte telomere length and mortality in humans.** *J Gerontol A-Biol* 2013.
35. De Angelis PM, Clausen OPF, Schjolberg A, Stokke T: **Chromosomal gains and losses in primary colorectal carcinomas detected by CGH and their associations with tumour DNA ploidy, genotypes and phenotypes.** *Br J Cancer* 1999, **80**(3–4):526–535.
36. Aust DE, Willenbucher RF, Terdiman JP, Ferrell LD, Chang CG, Moore DH 2nd, Molinaro-Clark A, Baretton GB, Loehrs U, Waldman FM: **Chromosomal alterations in ulcerative colitis-related and sporadic colorectal cancers by comparative genomic hybridization.** *Hum Pathol* 2000, **31**(1):109–114.
37. Salk JJ, Salipante SJ, Risques RA, Crispin DA, Li L, Bronner MP, Brentnall TA, Rabinovitch PS, Horwitz MS, Loeb LA: **Clonal expansions in ulcerative colitis identify patients with neoplasia.** *ProcNatlAcadSciUSA* 2009, **106**(49):20871–20876.
38. Burmer GC, Rabinovitch PS, Haggitt RC, Crispin DA, Brentnall TA, Kolli VR, Stevens AC, Rubin CE: **Neoplastic progression in ulcerative colitis: histology, DNA content, and loss of a p53 allele.** *Gastroenterology* 1992, **103**(5):1602–1610.
39. Salk JJ, Bansal A, Lai LA, Crispin DA, Ussakli CH, Horwitz MS, Bronner MP, Brentnall TA, Loeb LA, Rabinovitch PS, *et al*: **Clonal expansions and short telomeres are associated with neoplasia in early-onset, but not late-onset, ulcerative colitis.** *Inflamm Bowel Dis* 2013, **19**(12):2593–2602.
40. Sipos F, Galamb O, Herszenyi L, Molnar B, Solymosi N, Zagoni T, Berczi L, Tulassay Z: **Elevated insulin-like growth factor 1 receptor, hepatocyte growth factor receptor and telomerase protein expression in mild ulcerative colitis.** *Scand J Gastroenterol* 2008, **43**(3):289–298.
41. Harbo M, Koelvraa S, Serakinci N, Bendix L: **Telomere dynamics in human mesenchymal stem cells after exposure to acute oxidative stress.** *DNA Repair (Amst)* 2012, **11**(9):774–779.
42. Seril DN, Liao J, Yang GY, Yang CS: **Oxidative stress and ulcerative colitis-associated carcinogenesis: studies in humans and animal models.** *Carcinogenesis* 2003, **24**(3):353–362.
43. Roessner A, Kuester D, Malfertheiner P, Schneider-Stock R: **Oxidative stress in ulcerative colitis-associated carcinogenesis.** *Pathol Res Pract* 2008, **204**(7):511–524.
44. Christensen R, Alsner J, Brandt Sorensen F, Dagnaes-Hansen F, Kolvraa S, Serakinci N: **Transformation of human mesenchymal stem cells in radiation carcinogenesis: long-term effect of ionizing radiation.** *Regen Med* 2008, **3**(6):849–861.

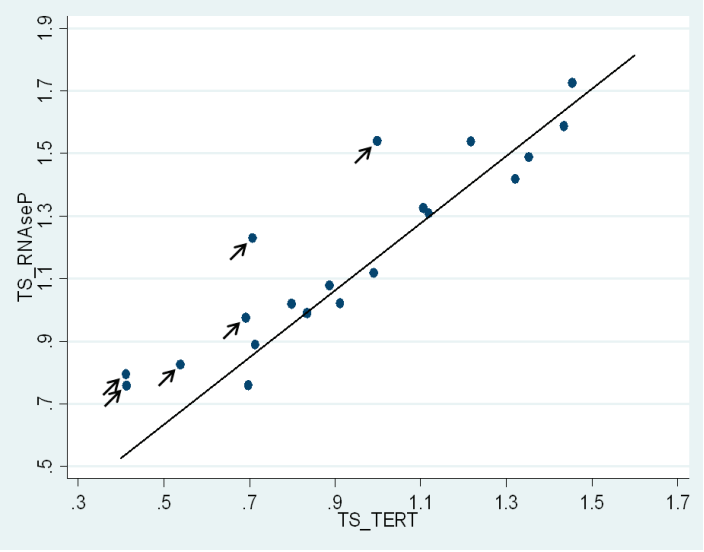
doi:10.1186/1471-230X-14-8
Cite this article as: Friis-Ottessen *et al.*: Telomere shortening correlates to dysplasia but not to DNA aneuploidy in longstanding ulcerative colitis. *BMC Gastroenterology* 2014 **14**:8.

Additional files:

Supplementary figure:
Mean telomere length estimated with two different single copy genes (SCG).
The relation between mean telomere estimated with the regular SCG RNAseP (TS_RNAseP) and the mean telomere length with the alternative TERT gene (TS_TERT). There is correlation in all samples but six (marked with arrows). These six samples were all from progressors and all are aneuploid. Of the six lesions four were adenocarcinomas, one was dysplastic and one was a non-dysplastic lesion.

Supplementary table:
Mean and p-values from t-tests.
Mean values and p-values of t-tests comparing progressors to nonprogressors, and comparing the different parameters from the progressor colons. Results are reported for U-STELA and for two different mean telomere analyses. One was using RNAseP as SCG, the other using TERT. Provided as a pdf-file.

Supplementary figure:



Supplementary table 1:

		U-STELA		Mean (RNAseP)		Mean (TERT)	
TOTAL UC COLECTOMIES		Progressors vs. non-progressors		Progressors vs. non-progressors		Progressors vs. non-progressors	
	N	51	27	69	22	67	22
	Mean	4.24	3.06	1.26	1.31	1.11	1.29
	p	<0.001		0.56		0.08	
		Non-dysplastic, diploid progressor lesions vs nonprogressors					
		Progressors vs. non-progressors		Progressors vs. non-progressors		Progressors vs. non-progressors	
	N	24	27	25	22	24	22
	Mean	3.96	3.06	1.43	1.31	1.29	1.26
	p	<0.001		0.35		0.83	
PROGRESSOR COLECTOMIES		Diploid vs. aneuploid		Diploid vs. aneuploid		Diploid vs. aneuploid	
	N	39	12	49	20	47	20
	Mean	4.19	4.39	1.29	1.18	1.13	0.93
	p	0.46		0.27		0.04	
		Non-dysplasia vs. Dysplasia*		Non-dysplasia vs. dysplasia*		Non-dysplasia vs. Dysplasia*	
	N	28	23	32	37	31	36
	Mean	3.97	4.57	1.41	1.11	1.22	0.93
	p	0.007		<0.001		<0.001	
		Only diploid lesions					
		Non-dysplasia vs. Dysplasia*		Non-dysplasia vs. Dysplasia*		Non-dysplasia vs. Dysplasia*	
	N	24	15	25	24	24	23
	Mean	3.96	4.57	1.43	1.14	1.26	0.99
	p	0.03		0.007		0.008	

*dysplasia includes indefinite for dysplasia, LGD, HGD and adecocarcinoma

II

TP53/p53 alterations and Aurora A expression in progressor and nonprogressor colectomies from patients with longstanding ulcerative colitis.

Mariann Friis-Ottessen[1], Espen Burum-Auensen[2], Aasa R. Schjølberg[3], Per Olaf Ekstrøm[4], Solveig N. Andersen[5], Ole Petter Clausen[3], Paula M De Angelis[1]

[1]Division of Diagnostics and Intervention, Department of Pathology, Oslo University Hospital, Rikshospitalet, Oslo, Norway
[2] Medivir Norge, Nydalsveien 33, Postboks 4814 Nydalen, 0484 Oslo
[3]Department of Pathology, University of Oslo, Oslo, Norway
[4] Division for Surgery and Cancer Medicine, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway
[5]Department of Pathology, Akershus University Hospital, Division of Medicine and Laboratory Sciences, University of Oslo, Oslo, Norway

Correspondence to:

Mariann Friis-Ottessen: mariann.friis-ottessen@rr-research.no
OUS HF Rikshospitalet, Postboks 4950 Nydalen
0424 Oslo, Norway
Tel.: + 47-23071487 Fax.: +47-23071511

Espen Burum-Auensen: eauensen@online.no

Aasa Rambæk Schjølberg: aasa.schjolberg@oslo-universitetssykehus.no

Per Olaf Ekstrøm: pok@rr-research.no

Solveig Norheim-Andersen: s.n.andersen@medisin.uio.no

Ole Petter Clausen: oclausen@ous-hf.no

Paula De Angelis: Paula.DeAngelis@rr-research.no

Key words:

Ulcerative colitis, aneuploidy, dysplasia, immunohistochemistry, p53, Aurora A

Running title:

Aurora A and p53 are in a relationship – it's complicated, by Mariann Friis-Ottessen

Abstract:

Aneuploidy is a common feature in the colonic mucosa of patients suffering from the inflammatory bowel disease ulcerative colitis, and often precedes the development of dysplasia and cancer. Aneuploidy is assumed to be caused by missegregation of chromosomes during mitosis, often due to a faulty spindle assembly checkpoint. p53 is a tumour suppressor protein known to regulate the spindle assembly checkpoint, and is frequently mutated in aneuploid cells. Aurora A is a presumed oncoprotein, also involved in regulation of the spindle assembly checkpoint.

We examined the mutational frequency of *TP53* and protein levels of p53 in a set of 20 progressor and 10 nonprogressor colectomies from patients suffering from longstanding ulcerative colitis. In the same material we re-examined previously published immunohistochemical data on Aurora A expression.

Levels of Aurora A were re-examined with regard to DNA-ploidy status and dysplasia within the progressors, as well as in relation to p53 accumulation and *TP53* mutational status.

We detected p53 accumulation only within the progressor colectomies, where it could be followed back to 12 years prior to colectomies in biopsies. *TP53* mutations were detected in both progressors and nonprogressors. Expression levels of Aurora A were similar in progressors and nonprogressors. Within progressors however, low levels of Aurora A were associated with areas of DNA aneuploidy as well as with increasing degrees of dysplasia.

Our results indicate that alterations in p53 may be an early biomarker of a progressor colon, and that p53 is accumulated early in UC-related carcinogenesis. Furthermore, reduced Aurora A expression is associated with development of DNA aneuploidy as well as with dysplasia in UC progressors.

Introduction:

Ulcerative colitis (UC) is an inflammatory bowel disease that predisposes for colorectal cancer. The risk of developing malignancies increases with disease duration, and also with age at disease onset (1). Malignant development in UC is a multistep progression through inflammation, regeneration, and dysplasia leading to adenocarcinoma. The process also includes a number of important molecular changes. The colonic mucosa of UC-patients may harbour severe molecular abnormalities due to chromosomal instability (CIN) leading to DNA aneuploidy. Aneuploidy is regarded as an early event in the malignant development of UC (2), and may be present in both dysplastic as well as non-dysplastic colonic mucosa (3, 4).

Aneuploidy in UC relates to disease duration (5-8), and is regarded as an independent risk factor for the development of adenocarcinoma in UC (9, 10). The consequences of aneuploidy in non-neoplastic cells are growth arrest or cell death, but is in UC suggested to be a precursor of future malignancies (6). More than half of the colorectal adenocarcinomas developing from UC present DNA-aneuploidy (7, 9), and it is thus considered a major contributor to the neoplastic phenotype (11, 12).

Aneuploidy can refer to either structural errors or copy-number errors in chromosomes, and aneuploid cells usually will contain a combination of these two errors. Structural errors are most likely products of chromosomal breakage, and whereas multiple mechanisms can underlie chromosomal breakage (13-15), copy number errors are achieved mainly through errors in chromosomal segregation (16, 17). The spindle checkpoint (or mitotic checkpoint) is crucial for the separation of chromosomes to both daughter cells during mitosis. It is a complex signalling cascade that will arrest mitosis upon faulty alignment of chromosomes or

if the spindle fails to attach to kinetochores properly (18-20). A dysfunctional spindle checkpoint is considered to be a major cause of aneuploidy in malignancies. (16, 17, 21).

p53 is a tumour suppressor protein with multiple functions in the regulation of the cell cycle and chromosomal stabilization (22). In cancers, there are often mutations and/or loss of heterozygosity in the *TP53* gene, resulting in loss of function. In UC-related carcinogenesis, evidence points to inactivation of p53 as being a relatively early event (22-24), whereas it is considered a late event in the development of sporadic colorectal cancers (25). p53 and Aurora A are reportedly involved in a mitotic feedback loop: p53 is proposed to be a negative regulator of Aurora A expression, whereas Aurora A can phosphorylate p53, rendering it incapable of binding to DNA, or marking it for degradation (22, 26-28). If wild-type p53 is assumed to be a negative regulator of the mitotic spindle kinase Aurora A (22, 29), loss of functional p53 may have serious implications for regulation of the spindle checkpoint. Loss of wild-type p53 function may result in centrosome amplification, faulty chromosomal segregation, and aneuploidy. In the absence of *TP53* mutations, accumulation of p53 in a UC-colon can also be due to a programmed p53 response to various reactive oxidative species present in inflamed tissue (30).

Overexpression of Aurora A is implicated in abnormal centrosome amplification and in abrogation of the spindle checkpoint (31). The gene coding for Aurora A is located on 20q13.2, a chromosomal arm frequently amplified in solid tumours, including colorectal tumours (32). The expression of Aurora A is reported to be elevated in several tumour types (33, 34), as well as in the colonic mucosa of UC-patients (35).

We have assessed both the mutational frequency of *TP53* and the protein levels of p53 in a set of colectomies from patients suffering from longstanding UC. We also re-evaluated previously-published data from Aurora A expression assessed by immunohistochemical stainings in the same colectomies (35). The colectomies were stratified as progressors and nonprogressors, as previously presented (36, 37). Within the progressors we assessed the results from Aurora A in association with DNA ploidy-status and advancing degrees of dysplasia, as well as with protein levels of p53 and *TP53* mutation status.

Materials and methods:

UC-colectomies and patients:

Thirty patients suffering from longstanding UC were included in this report. All patients had suffered from UC for more than ten years before colectomy; some as long as 30 years. Patients also varied widely with respect to age at the time when symptoms were first presented (from 10 to 60 years old).

The colectomy specimens have previously been described (35-37). We divided the colectomies into progressors and nonprogressors, revealing 10 nonprogressors that did not present any dysplastic lesions or DNA aneuploidy, and 20 progressors that all presented at least one area of dysplasia/cancer, where the majority of cases also presented lesions with DNA aneuploidy.

At least eight locations from each colectomy were examined, and within the progressors we found 83 non-dysplastic areas, 31 areas indefinite for dysplasia, 29 areas with dysplasia, and 8 adenocarcinomas.

Eighteen non-dysplastic and 20 dysplastic areas revealed DNA aneuploidy. The aneuploid, dysplastic areas included 8 areas of indefinite dysplasia and 5 adenocarcinomas. By definition all nonprogressor lesions were diploid and non-dysplastic.

Detailed distributions of dysplasia and aneuploid lesions within the progressors have previously been presented (35, 37).

Tissue micro-array evaluation:

Tissue microarrays (TMAs) from eight locations within each colon were made using a Beecher tissue microarrayer as described earlier (35). Core size was 0.6 mm. All cores have been previously evaluated by an experienced pathologist (OPFC). At least two tissue cores from each mucosal region were sampled.

TMAs do not consistently display full colonic crypts as do whole sections. p53 staining was performed on whole sections since detectable accumulation of p53 is heterogeneous (positively- and negatively-stained areas in the same section). If tissue cores for TMAs were sampled from areas negative for p53, this might lead to an increased number of false negatives. The expression of Aurora A was homogeneous (staining was evenly-distributed throughout the section), thus TMAs were regarded as reliable for estimation of Aurora A protein expression.

Immunohistochemistry (IHC):

Immunohistochemical stainings for p53 and Aurora A were performed as described in our previous publications (35, 38, 39).

p53 accumulation was assessed microscopically, by manually counting positive nuclei in whole sections, as presented previously (38). At least 1200 nuclei were counted, and a section was scored as positive for p53 if more than 5% of the cells in a section showed nuclear staining as previously presented by our research group (40).

Aurora A expression was assessed from TMAs. Aurora A protein expression was defined for each sample as the percentage of positive cells out of at least 300 randomly selected mucosal epithelial cells from each included tissue core. Staining of Aurora A and p53 are presented in figure 1 A and B. With increasing degree of dysplasia, an increasing amount of cells with nucleic positivity of Aurora A also presented cytoplasmic staining.

p53 mutation analysis:

Mutation analysis for *TP53* exons 5-8 was performed by cycling temperature capillary electrophoresis (CTCE) (41, 42). This procedure detects the presence of mutations; we did not sequence the mutation-positive cases in order to determine the actual mutation.

The primer sequences for the mutation analyses are previously presented by Bjørheim and colleagues (43).

Statistics:

p53/*TP53* correlations were performed in cross tabulation and assessed by Pearson's Chi-square test. Assessment of Aurora A protein levels in association with DNA-ploidy status, mucosal morphology and p53 accumulation, as well as *TP53* mutational status, was performed using a multilevel model compensating for patient differences, as each patient included in this study contributed with more than one biopsy. A linear mixed model (LMM), with restricted maximum likelihood (REML) estimations, and a Bonferroni post hoc test were used. Tests were performed in PASW statistics 18 (Chicago, IL, USA). All tests were two-sided and a p-level of 0.05 denoted significance.

Results:

p53 immunohistochemistry:

No accumulation of p53 was detected within the 10 nonprogressor colectomies.

Of the 20 progressor colectomies, 60 % (12/20) harboured areas with accumulation of p53, but no colectomy specimens showed p53 accumulation in all eight lesions.

The 20 progressors had a total of 130 lesions available for p53 assessment, and 20.8 % (27/130) of the lesions were positive for p53 accumulation. Within the positive lesions 22.2 % (6/27) also contained aneuploid populations. In precolectomy biopsies from patients with colectomies positive for p53 accumulation, we detected p53 accumulation up to 14 years prior to colectomy. A summary of colectomy lesions positive for p53 accumulation, including DNA-ploidy status and mucosal morphology, is presented in table 1.

Table 1: IHC results from p53 positive lesions (N = 27) in progressors.

	>5% p53 staining	
	diploid	aneuploid
non-dysplasia	8	0
Indef. dysplasia	5	1
dysplasia	5	2
adenocarcinoma	3	3

Mutation analysis of the *TP53* gene (exons 5–8)

TP53 mutations were found in both progressors and nonprogressors. 70% (7/10) of the nonprogressors and 55% (11/20) of the progressors harboured areas with mutations in one of the *TP53* mutational hotspots (exons 5-8).

Of the 70 nonprogressor lesions available for *TP53* mutation analysis 20% (14/70) had a *TP53* mutation. The 20 progressor colectomies yielded 129 lesions available for *TP53* mutation analysis. From these 129 lesions 11.6% (15/129) had mutation in one of the mutational hotspots examined (exons 5-8). 20.8% (9/15) of the mutated lesions also had aneuploid cell populations. Table 2 shows a summary of lesions with *TP53* mutations, with DNA ploidy and mucosal morphology.

Table 2: UC-colectomy lesions with mutated *TP53*.

		mutated *TP53*	
Colon	Morphology	diploid	aneuploid
nonprogressors	non-dysplasia	14	0
progressors	non-dysplasia	2	4
	Indef. dysplasia	3	2
	dysplasia	0	1
	adenocarcinoma	1	2

No correlation was detected between mutation in *TP53* and accumulation of p53 in this material. Three progressor lesions had both a *TP53* mutation and accumulation of p53.

All three lesions originated from separate colons, and included two adenocarcinomas, and one lesion indefinite for dysplasia. One of the adenocarcinomas was also aneuploid.

Aurora A expression in UC progressors and nonprogressors:

We have previously published that expression of Aurora A in UC mucosa is elevated compared to non-UC control samples (35).

Aurora A expression did not differ between progressors and nonprogressors, neither when including all types of progressor lesions, nor when only diploid, non-dysplastic progressor lesions were included.

Within the progressors we found a significant association between Aurora A and DNA ploidy-status ($p = 0.020$), with lower levels of Aurora A present in lesions harbouring aneuploid populations (figure 2).

Aurora A expression within the progressor lesions decreased with increasing severity of dysplasia, but when accounting for patient variation this was not statistically significant. The lowest values of Aurora A expression were seen within high-grade dysplasia. Adenocarcinomas harboured increased amounts of Aurora A expression (figure 3a). As only six lesions were diagnosed as high-grade dysplasia, these were combined with low-grade dysplasia for statistical purposes. Excluding the 6 colectomies harbouring adenocarcinomas, a significant decrease in Aurora A expression associating with increasing degrees of dysplasia was seen in the 14 remaining colectomies ($p = 0.025$) (figure 3b).

Expression of Aurora A associated with p53 accumulation/*TP53* mutation

Colectomies harbouring at least one lesion with p53 accumulation displayed decreased levels of Aurora A, compared to colectomies with no p53-accumulation (figure 4), however not to a significant degree when inter-patient differences were accounted for.

Aurora A expression was not significantly associated with p53 mutation status in our material.

Discussion:

It has long been known that protein levels of Aurora A are up-regulated in most solid tumours (31, 44), linked to CIN and aneuploidy (33, 45) and associated with poor prognosis (46). It is also known that Aurora A is mapped to chromosome 20q13.2, a region highly amplified in for example sporadic colorectal cancers (32). UC-colonic mucosa is subjected to rapid cell division (47), and high levels of oxidative stress (48), regardless of its status as progressor or nonprogressor. Oxidative stress has been shown to induce spindle checkpoint override in cell lines, as it can inhibit the anaphase-promoting complex/cyclosome (APC/C) (49). Aurora A in normal functioning cells is targeted by APC/C for degradation during late mitosis, a function essential for mitotic exit. Persistent Aurora A might be able to prolong the anaphase and induce separation of chromatids (50). Both progressors and nonprogressors in our material presented elevated levels of Aurora A compared to non-UC control samples, but only progressors revealed dysplastic development and DNA-ploidy changes. This may imply that the general increase in Aurora A levels seen in UC colonic mucosa is consistent with enhanced spindle checkpoint activity as a natural response to an accelerated cellular proliferation as well as elevated levels of oxidative stress. Other factors however, are also most likely needed to override the checkpoint function, inducing CIN and DNA aneuploidy.

We have previously presented findings of similar levels of hTERT protein expression and equal shortening of mean telomere length in the colonic mucosa of progressors compared to nonprogressors from the same UC-patient material (36, 37). These results are in accordance with UC being a disease that accelerates aging of the colonic mucosa (51), but these parameters are unable to differentiate a progressor from a nonprogressor UC-colon.

Likewise, our results indicate that Aurora A expression is not an ideal biomarker for differentiating progressor from nonprogressor UC-colons.

TP53 mutations were detected in both progressors and nonprogressors, consistent with the observation that the frequency of *TP53* mutation increases after at least 10 years of UC duration, and without association to malignancies (52) and the observation that *TP53* mutations are frequent in inflamed tissue of UC colons (53). Interestingly, the majority of mutations were found within the nonprogressors; however, no p53 accumulation was seen in the nonprogressors. The reason for this is unclear. Lack of such correlation has previously been shown at the single crypt level in UC (54). Lack of detectable p53 maybe due to nonsense mutations and premature stop codons, rather than missense mutations; since with a missense mutation, accumulation of p53 is to be expected. It has been observed that less than 20% of *TP53* mutations of human cancers are nonsense mutations or stop codon mutations (55, 56). It has also been observed that mutation of *TP53* happens prior to loss of heterozygosity in UC colonic mucosa in UC progressors (57), and our results may be indicative of an early mutation of a single *TP53* allele, whereas the remaining allele provides functional p53 in these nonprogressors. Since we did not sequence the cases positive for *TP53* mutation, this aspect of our study remains unclear.

Pre-colectomy biopsies from the patients included in our study made it possible to track p53 positivity retrospectively. From the 11 patients displaying p53-accumulation in precolectomy biopsies: all were found in patients with colons harbouring progressor traits at colectomy. Six cases had indeed developed adenocarcinomas. The finding that only progressors showed p53 accumulation indicates that p53 accumulation may be a potential biomarker of a progressor colon, consistent with previous reports of p53 expression associating with dysplastic development in UC (58-60).

The expression levels of Aurora A within the progressors did not differ to a statistically significant degree when comparison was made between the advancing degrees of dysplasia including adenocarcinomas. However; when the six colectomies with cancers detected were removed, a statistically significant decrease in Aurora A expression showed associations with increasing levels of dysplasia. Adenocarcinomas presented elevated levels of Aurora A compared to dysplastic lesions (figure 3A). In addition, Aurora A was also significantly associated with DNA aneuploidy within the progressors (figure 2). This relationship was masked when progressors and nonprogressors were combined (35). In our material the aneuploid lesions had decreased amounts of Aurora A compared to diploid lesions, again with exception of adenocarcinomas, where the aneuploid cancers had elevated levels of Aurora A compared to the diploid cancers (data not shown). As colon cancers have been shown to harbour high levels of 20q-amplifications (32), a trait not often seen within noncancerous UC-mucosa (2), this might be an explanation for the elevated levels of Aurora A in adenocarcinomas compared to the dysplastic lesions in our material.

Recently a study of Aurora A expression in dysplasia and cancer in gastric mucosa showed increased levels of Aurora A in dysplastic gastric lesions (61). As this is contrary to our findings in UC-mucosa, it may indicate that different mechanisms are involved in dysplastic development in the colonic and the gastric mucosa.

p53 has been shown to be an important negative regulator of Aurora A. Loss of p53 can lead to abnormal regulation of Aurora A and dysregulated mitosis (29, 62, 63). An increase of Aurora A may induce a protective mechanism, oncogene-induced senescence, against malignant development, possibly dependent on loss of functional p53 (64-66) . As our data show decreased Aurora A expression in areas harbouring aneuploid populations, it might be

possible that the development of CIN and aneuploidy is necessary to overcome this protection.

Our results showing that nonprogressor lesions with wt *TP53* have higher Aurora A levels than mutated *TP53* nonprogressor lesions, although not a significant difference when inter-patient differences were re accounted for (data not shown), are also consistent with this idea.

Wild-type p53 is difficult to detect in normal unstressed cells. Detection of p53 protein becomes possible due to the extended half-life of a mutated, non-functioning protein or by stabilisation of p53 as a natural response to for example cellular stress and inflammation (30, 67). p53 accumulation is also a known response to excess shortening of telomeres (68, 69). As we have previously shown that the mucosa of UC-progressor cases harbours significantly more ultra-short telomeres than found within the nonprogressor cases (36), this could indicate that telomeric repeat-induced activation of p53 is a possibility in our progressor cases. The lack of detectable p53 in nonprogressors can perhaps also indicate that the elevated levels of Aurora A in the nonprogressors are phosphorylating p53, targeting it for degradation. This is also consistent with a previous publication that reported that Aurora A phosphorylates p53 at Ser315, leading to MDM2-mediated ubiquitination and degradation of p53 (62).

Our findings of no p53 accumulation detected in the nonprogressors differ from previous reports (70), and can perhaps be due to our definition of a nonprogressor: we have included only non-dysplastic patients (described as having regenerative or inflamed mucosa by two experienced pathologists (OPFC and SNA)) with no detectable DNA-aneuploidy. We chose this definition as it has been shown that even UC-patients with only one lesion indefinite for dysplasia, or with DNA aneuploidy alone, can develop adenocarcinoma (71, 72).

Conclusion:

Our findings indicate that p53 accumulation may be a good biomarker for progressor UC-cases, as no accumulation was detected in nonprogressors, and the progressors showed p53 accumulation in biopsies collected several years prior to colectomy. Expression of Aurora A did not differ between progressor and nonprogressor UC-colectomies. Within the progressor cases levels of Aurora A were decreased in association with both aneuploidy and dysplasia, but increased in adenocarcinomas. p53 and Aurora A appear to regulate each other in a different manner in progressors and nonprogressors.

Ethical Considerations:

Use of this material for research purposes has ethical approval from the Regional Ethical Committee, REK S-06062.

Competing interests:

This work was made possible by the generous funding from South-Eastern Norway Regional Health Authority and by Stiftelsen UNI. These organisations had no role in collecting, analysing, or interpreting the data or in writing the report.

References:

1. Salk JJ, Bansal A, Lai LA*, et al*: Clonal Expansions and Short Telomeres Are Associated with Neoplasia in Early-onset, but not Late-onset, Ulcerative Colitis. Inflamm Bowel Dis 19: 2593-2602, 2013.

2. Willenbucher RF, Aust DE, Chang CG*, et al*: Genomic instability is an early event during the progression pathway of ulcerative-colitis-related neoplasia. Am J Pathol 154: 1825-1830, 1999.

3. Rubin CE, Haggitt RC, Burmer GC*, et al*: DNA aneuploidy in colonic biopsies predicts future development of dysplasia in ulcerative colitis. Gastroenterology 103: 1611-1620, 1992.

4. Rabinovitch PS, Dziadon S, Brentnall TA*, et al*: Pancolonic chromosomal instability precedes dysplasia and cancer in ulcerative colitis. Cancer Res. 59: 5148-5153, 1999.

5. Hammarberg C, Slezak P and Tribukait B: Early detection of malignancy in ulcerative colitis. A flow-cytometric DNA study. Cancer 53: 291-295, 1984.

6. Fozard JB, Quirke P, Dixon MF, Giles GR and Bird CC: DNA aneuploidy in ulcerative colitis. Gut 27: 1414-1418, 1986.

7. Meling GI, Clausen OP, Bergan A, Schjolberg A and Rognum TO: Flow cytometric DNA ploidy pattern in dysplastic mucosa, and in primary and metastatic carcinomas in patients with longstanding ulcerative colitis. Br J Cancer 64: 339-344, 1991.

8. Meyer KF, Nause SL, Freitag-Wolf S*, et al*: Aneuploidy characterizes adjacent non-malignant mucosa of ulcerative colitis-associated but not sporadic colorectal carcinomas: a matched-pair analysis. Scand J Gastroenterol 48: 679-687, 2013.

9. Gerling M, Meyer KF, Fuchs K*, et al*: High Frequency of Aneuploidy Defines Ulcerative Colitis-Associated Carcinomas: A Comparative Prognostic Study to Sporadic Colorectal Carcinomas. Ann Surg 252: 74-83, 2010.

10. Gerling M, Nousiainen K, Hautaniemi S*, et al*: Aneuploidy-associated gene expression signatures characterize malignant transformation in ulcerative colitis. Inflamm Bowel Dis 19: 691-703, 2013.

11. Lengauer C, Kinzler KW and Vogelstein B: Genetic instabilities in human cancers. Nature 396: 643-649, 1998.

12. Davoli T and de Lange T: The causes and consequences of polyploidy in normal development and cancer. Annu Rev Cell Dev Biol 27: 585-610, 2011.

13. Londono-Vallejo JA: Telomere instability and cancer. Biochimie 90: 73-82, 2008.

14. Cheung AL and Deng W: Telomere dysfunction, genome instability and cancer. Front Biosci. 13: 2075-2090, 2008.

15. Kong CM, Lee XW and Wang X: Telomere shortening in human diseases. FEBS J 280: 3180-3193, 2013.

16. Kops GJ, Weaver BA and Cleveland DW: On the road to cancer: aneuploidy and the mitotic checkpoint. Nat Rev Cancer 5: 773-785, 2005.

17. Bharadwaj R and Yu H: The spindle checkpoint, aneuploidy, and cancer. Oncogene 23: 2016-2027, 2004.

18. Musacchio A and Salmon ED: The spindle-assembly checkpoint in space and time. Nat Rev Mol Cell Biol 8: 379-393, 2007.

19. Musacchio A and Hardwick KG: The spindle checkpoint: structural insights into dynamic signalling. Nat Rev Mol Cell Biol 3: 731-741, 2002.

20. Kops GJ, Foltz DR and Cleveland DW: Lethality to human cancer cells through massive chromosome loss by inhibition of the mitotic checkpoint. Proc Natl Acad Sci U S A 101: 8699-8704, 2004.

21. Weaver BA and Cleveland DW: Decoding the links between mitosis, cancer, and chemotherapy: The mitotic checkpoint, adaptation, and cell death. Cancer Cell 8: 7-12, 2005.

22. Aylon Y and Oren M: p53: guardian of ploidy. Mol Oncol 5: 315-323, 2011.

23. Sato A and MacHinami R: p53 immunohistochemistry of ulcerative colitis-associated with dysplasia and carcinoma. Pathol Int 49: 858-868, 1999.

24. Klump B, Holzmann K, Kuhn A*, et al*: Distribution of cell populations with DNA aneuploidy and p53 protein expression in ulcerative colitis. Eur J Gastroenterol Hepatol 9: 789-794, 1997.

25. Baker SJ, Preisinger AC, Jessup JM*, et al*: p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. Cancer Res 50: 7717-7722, 1990.

26. Mao JH, Wu D, Perez-Losada J*, et al*: Crosstalk between Aurora-A and p53: Frequent deletion or downregulation of Aurora-A in tumors from p53 null mice. Cancer Cell 11: 161-173, 2007.

27. Ha GH and Breuer EK: Mitotic Kinases and p53 Signaling. Biochem Res Int 2012: 195903, 2012.

28. Katayama H, Wang J, Treekitkarnmongkol W*, et al*: Aurora kinase-A inactivates DNA damage-induced apoptosis and spindle assembly checkpoint response functions of p73. Cancer Cell 21: 196-211, 2012.

29. Wu CC, Yang TY, Yu CTR*, et al*: p53 negatively regulates Aurora A via both transcriptional and posttranslational regulation. Cell Cycle 11: 3433-3442, 2012.

30. Forrester K, Ambs S, Lupold SE*, et al*: Nitric oxide-induced p53 accumulation and regulation of inducible nitric oxide synthase expression by wild-type p53. Proc Natl Acad Sci U S A 93: 2442-2447, 1996.

31. Nikonova AS, Astsaturov I, Serebriiskii IG, Dunbrack RL, Jr. and Golemis EA: Aurora A kinase (AURKA) in normal and pathological cell division. Cell Mol Life Sci 70: 661-687, 2013.

32. De Angelis PM, Clausen OPF, Schjolberg A and Stokke T: Chromosomal gains and losses in primary colorectal carcinomas detected by CGH and their associations with tumour DNA ploidy, genotypes and phenotypes. British Journal of Cancer 80: 526-535, 1999.

33. Zhou H, Kuang J, Zhong L*, et al*: Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. Nat Genet 20: 189-193, 1998.

34. Marumoto T, Zhang D and Saya H: Aurora-A - a guardian of poles. Nat Rev Cancer 5: 42-50, 2005.

35. Burum-Auensen E, De Angelis PM, Schjolberg AR, Roislien J, Andersen SN and Clausen OP: Spindle proteins Aurora A and BUB1B, but not Mad2, are aberrantly expressed in dysplastic mucosa of patients with longstanding ulcerative colitis. J Clin Pathol 60: 1403-1408, 2007.

36. Friis-Ottessen M, Bendix L, Kolvraa S, Norheim-Andersen S, De Angelis PM and Clausen OP: Telomere shortening correlates to dysplasia but not to DNA aneuploidy in longstanding ulcerative colitis. BMC Gastroenterol 14: 8, 2014.

37. Friis-Ottessen M, De Angelis PM, Schjolberg AR, Andersen SN and Clausen OP: Reduced hTERT protein levels are associated with DNA aneuploidy in the colonic mucosa of patients suffering from longstanding ulcerative colitis. Int J Mol Med 2014.

38. Schjolberg AR, Clausen OPF, Burum-Auensen E and De Angelis PM: Aneuploidy Is Associated with TP53 Expression but not with BRCA1 or TERT Expression in Sporadic Colorectal Cancer. Anticancer Research 29: 4381-4387, 2009.

39. Burum-Auensen E, De Angelis PM, Schjolberg AR, Kravik KL, Aure M and Clausen OP: Subcellular localization of the spindle proteins Aurora A, Mad2, and BUBR1 assessed by immunohistochemistry. J Histochem Cytochem 55: 477-486, 2007.

40. Clausen OP, Lothe RA, Borresen-Dale AL*, et al*: Association of p53 accumulation with TP53 mutations, loss of heterozygosity at 17p13, and DNA ploidy status in 273 colorectal carcinomas. Diagn Mol Pathol 7: 215-223, 1998.

41. Ekstrom PO, Warren DJ and Thilly WG: Separation principles of cycling temperature capillary electrophoresis. Electrophoresis 33: 1162-1168, 2012.

42. Ekstrom PO, Khrapko K, Li-Sucholeiki XC, Hunter IW and Thilly WG: Analysis of mutational spectra by denaturing capillary electrophoresis. Nat Protoc 3: 1153-1166, 2008.

43. Bjorheim J, Gaudernack G and Ekstrom PO: Mutation analysis of TP53 exons 5-8 by automated constant denaturant capillary electrophoresis. Tumour Biol 22: 323-327, 2001.

44. Bischoff JR, Anderson L, Zhu Y*, et al*: A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers. EMBO J 17: 3052-3065, 1998.

45. Baba Y, Nosho K, Shima K*, et al*: Aurora-A expression is independently associated with chromosomal instability in colorectal cancer. Neoplasia 11: 418-425, 2009.

46. Goos JA, Coupe VM, Diosdado B*, et al*: Aurora kinase A (AURKA) expression in colorectal cancer liver metastasis is associated with poor prognosis. Br J Cancer 109: 2445-2452, 2013.

47. Greco V, Lauro G, Fabbrini A and Torsoli A: Histochemistry of the colonic epithelial mucins in normal subjects and in patients with ulcerative colitis. A qualitative and histophotometric investigation. Gut 8: 491-496, 1967.

48. Roessner A, Kuester D, Malfertheiner P and Schneider-Stock R: Oxidative stress in ulcerative colitis-associated carcinogenesis. Pathol Res Pract 204: 511-524, 2008.

49. D'Angiolella V, Santarpia C and Grieco D: Oxidative stress overrides the spindle checkpoint. Cell Cycle 6: 576-579, 2007.

50. Floyd S, Pines J and Lindon C: APC/C Cdh1 targets aurora kinase to control reorganization of the mitotic spindle at anaphase. Curr Biol 18: 1649-1658, 2008.

51. Risques RA, Lai LA, Brentnall TA*, et al*: Ulcerative colitis is a disease of accelerated colon aging: evidence from telomere attrition and DNA damage. Gastroenterology 135: 410-418, 2008.

52. Lang SM, Stratakis DF, Heinzlmann M, Heldwein W, Wiebecke B and Loeschke K: Molecular screening of patients with long standing extensive ulcerative colitis: detection of p53 and Ki-ras mutations by single strand conformation polymorphism analysis and differential hybridisation in colonic lavage fluid. Gut 44: 822-825, 1999.

53. Hussain SP, Amstad P, Raja K*, et al*: Increased p53 mutation load in noncancerous colon tissue from ulcerative colitis: a cancer-prone chronic inflammatory disease. Cancer Res 60: 3333-3337, 2000.

54. Yoshida T, Mikami T, Mitomi H and Okayasu I: Diverse p53 alterations in ulcerative colitis-associated low-grade dysplasia: full-length gene sequencing in microdissected single crypts. J Pathol 199: 166-175, 2003.

55. van Schaik FD, Oldenburg B, Offerhaus GJ*, et al*: Role of immunohistochemical markers in predicting progression of dysplasia to advanced neoplasia in patients with ulcerative colitis. Inflamm Bowel Dis 18: 480-488, 2012.

56. Harris CC and Hollstein M: Clinical implications of the p53 tumor-suppressor gene. N Engl J Med 329: 1318-1327, 1993.

57. Brentnall TA, Crispin DA, Rabinovitch PS*, et al*: Mutations in the p53 gene: an early marker of neoplastic progression in ulcerative colitis. Gastroenterology 107: 369-378, 1994.

58. Wong NA, Mayer NJ, MacKell S, Gilmour HM and Harrison DJ: Immunohistochemical assessment of Ki67 and p53 expression assists the diagnosis and grading of ulcerative colitis-related dysplasia. Histopathology 37: 108-114, 2000.

59. Shigaki K, Mitomi H, Fujimori T*, et al*: Immunohistochemical analysis of chromogranin A and p53 expressions in ulcerative colitis-associated neoplasia: neuroendocrine differentiation as an early event in the colitis-neoplasia sequence. Hum Pathol 44: 2393-2399, 2013.

60. Harpaz N, Peck AL, Yin J*, et al*: p53 protein expression in ulcerative colitis-associated colorectal dysplasia and carcinoma. Hum Pathol 25: 1069-1074, 1994.

61. Katsha A, Soutto M, Sehdev V*, et al*: Aurora kinase A promotes inflammation and tumorigenesis in mice and human gastric neoplasia. Gastroenterology 145: 1312-1322 e1318, 2013.

62. Katayama H, Sasai K, Kawai H*, et al*: Phosphorylation by aurora kinase A induces Mdm2-mediated destabilization and inhibition of p53. Nat Genet 36: 55-62, 2004.

63. Vader G and Lens SM: The Aurora kinase family in cell division and cancer. Biochim Biophys Acta 1786: 60-72, 2008.

64. Bartkova J, Rezaei N, Liontos M*, et al*: Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature 444: 633-637, 2006.

65. Suram A, Kaplunov J, Patel PL*, et al*: Oncogene-induced telomere dysfunction enforces cellular senescence in human cancer precursor lesions. EMBO J 31: 2839-2851, 2012.

66. Zhang D, Shimizu T, Araki N*, et al*: Aurora A overexpression induces cellular senescence in mammary gland hyperplastic tumors developed in p53-deficient mice. Oncogene 27: 4305-4314, 2008.

67. Ashcroft M and Vousden KH: Regulation of p53 stability. Oncogene 18: 7637-7643, 1999.

68. Milyavsky M, Mimran A, Senderovich S*, et al*: Activation of p53 protein by telomeric (TTAGGG)n repeats. Nucleic Acids Res. 29: 5207-5215, 2001.

69. di Fagagna FD, Reaper PM, Clay-Farrace L*, et al*: A DNA damage checkpoint response in telomere-initiated senescence. Nature 426: 194-198, 2003.

70. Risques RA, Lai LA, Himmetoglu C*, et al*: Ulcerative colitis-associated colorectal cancer arises in a field of short telomeres, senescence, and inflammation. Cancer Res. 71: 1669-1679, 2011.

71. Gorfine SR, Bauer JJ, Harris MT and Kreel I: Dysplasia complicating chronic ulcerative colitis: is immediate colectomy warranted? Dis Colon Rectum 43: 1575-1581, 2000.

72. Ullman TA, Loftus EV, Jr., Kakar S, Burgart LJ, Sandborn WJ and Tremaine WJ: The fate of low grade dysplasia in ulcerative colitis. Am J Gastroenterol 97: 922-927, 2002.

Figure legends:

Figure 1: immunohistochemistry

Immunohistochemical staining of A) Aurora A and B) p53 in dysplastic UC colonic mucosa. Aurora A staining of non-dysplastic UC mucosa and non-UC control is previously presented (35). p53 staining illustrates heterogeneity, showing one positive and one negative crypt. Images are x400 magnification.

Figure 2: Aurora A expression and DNA-ploidy status

Levels of Aurora A expression in ulcerative colitis nonprogressors (all diploid) and in the diploid and aneuploid lesions from progressors. Within the progressors lesions harbouring aneuploid populations had significantly decreased levels of Aurora A expression compared to diploid lesions ($p = 0.020$).

Median values with 95% confidence interval.

Figure 3: Aurora A expression in progressor UC-colons with and without adenocarcinoma

Levels of Aurora A protein expression in lesions from A) 10 nonprogressor colons (white circle), and the degreasing levels of Aurora A with increasing degrees of dysplasia within the 20 progressor colons, including those harbouring adenocarcinomas, and B) the decreasing levels of Aurora A with increasing degrees of dysplasia in the 14 progressor colons remaining when excluding the colons harbouring adenocarcinomas (high-grade and low-grade dysplasia are combined) ($p = 0.025$). Star denotes significance at 0.05 levels, horizontal bar indicate overall difference between groups (as detected by LMM with Bonferroni post-test).

Median values with 95% confidence interval.

Figure 4: Aurora A expression and p53 accumulation

Aurora A expression in UC-progressor colons with lesions positive for p53 accumulation (>5 % positivity) and in progressor colons without any lesions displaying p53 accumulation.

Note the decreased expression of Aurora A in progressors without p53 accumulation, however not to a statistically significant degree when inter-patient differences are accounted for.

Median values with 95% confidence interval.

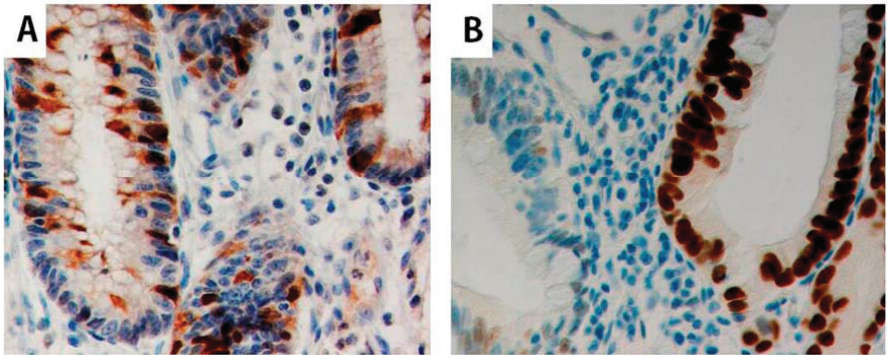


Figure 1

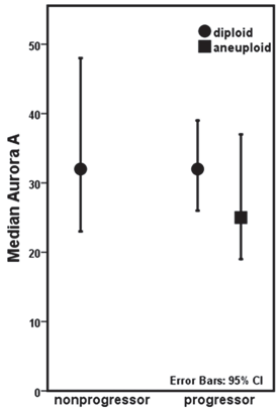

diploid
aneuploid
Median Aurora A
50
40
30
20
10
0
Error Bars: 95% CI
nonprogressor
progressor

Figure 2

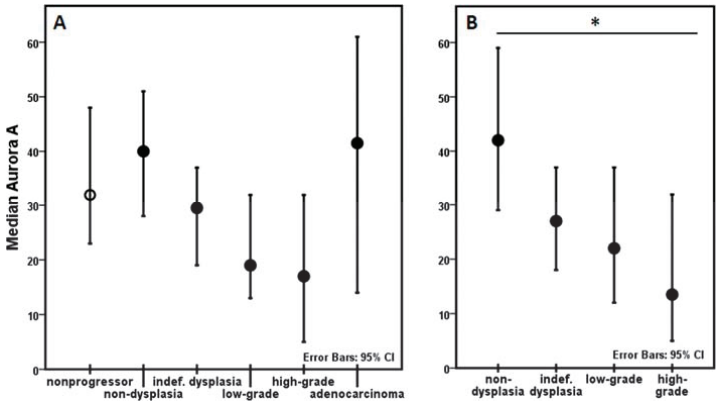

A
Median Aurora A
60
50
40
30
20
10
0
Error Bars: 95% CI
nonprogressor
non-dysplasia
indef. dysplasia
low-grade
high-grade
adenocarcinoma
B
*
60
50
40
30
20
10
0
Error Bars: 95% CI
non-
dysplasia
indef.
dysplasia
low-grade
high-
grade

Figure 3

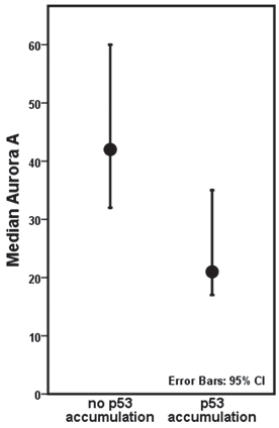

Median Aurora A
60
50
40
30
20
10
0
Error Bars: 95% CI
no p53 accumulation
p53 accumulation

Figure 4

III

Reduced hTERT protein levels are associated with DNA aneuploidy in the colonic mucosa of patients suffering from longstanding ulcerative colitis

MARIANN FRIIS-OTTESSEN[1], PAULA M. DE ANGELIS[1], AASA R. SCHJØLBERG[2], SOLVEIG N. ANDERSEN[3] and OLE PETTER F. CLAUSEN[1,2]

[1]Division of Diagnostics and Intervention, Department of Pathology, Oslo University Hospital, Rikshospitalet, 0424 Oslo; [2]Department of Pathology, University of Oslo, 0424 Oslo; [3]Department of Pathology, Akershus University Hospital, Division of Medicine and Laboratory Sciences, University of Oslo, 1474 Nordbyhagen, Norway

Received January 31, 2014; Accepted February 24, 2014

DOI: 10.3892/ijmm.2014.1708

Abstract. Longstanding ulcerative colitis (UC) is a disease of chronic inflammation of the colon. It is associated with the development of colorectal cancer through a multistep process including increasing degrees of dysplasia and DNA-ploidy changes. However, not all UC patients will develop these characteristics even during lifelong disease, and patients may therefore be divided into progressors who develop dysplasia or cancer, and non-progressors who do not exhibit such changes. In the present study, the amount of hTERT, the catalytic subunit of the enzyme telomerase, was estimated by using peroxidase immunohistochemistry (IHC) in a set of progressor and non-progressor UC colectomies. The protein levels in the colonic mucosa of the progressors and non-progressors were compared, and further comparisons between different categories of dysplastic development and to DNA-ploidy status within the progressors were made. Levels of hTERT were elevated in the colonic mucosa of the progressors and non-progressors when compared to non-UC control samples, but no difference was observed between the hTERT levels in the mucosa of progressors and non-progressors. The levels of hTERT associated with levels of Ki67 to a significant degree within the non-progressors. hTERT expression in lesions with DNA-aneuploidy were decreased as compared to diploid lesions, when stratified for different classes of colonic morphology. Our results indicate an association between hTERT protein expression and aneuploidy in UC-progressor colons, and also a possible protective mechanism in the association between hTERT and Ki67, against development of malignant features within the mucosa of a UC-colon.

Correspondence to: Ms. Mariann Friis-Ottessen, Division of Diagnostics and Intervention, Department of Pathology, Oslo University Hospital, OUS HF Rikshospitalet, Postboks 4950 Nydalen, 0424 Oslo, Norway
E-mail: mariann.friis-ottessen@rr-research.no

Key words: aneuploidy, dysplasia, immunohistochemistry, telomerase, ulcerative colitis

Introduction

Ulcerative colitis (UC) is an inflammatory bowel disease that is associated with an elevated risk of developing colorectal cancer, and it is estimated that 10% of patients suffering from UC for >10 years will develop colorectal cancer (1,2). The underlying pathogenesis is not fully known, but chronic inflammation distorts mucosal morphology and induces dysplasia and subsequently cancer. Carcinomas occur mainly after long-term illness of 8-10 years (3), and develop through low- and high-degree dysplasia. It is reported that patients presenting only one lesion of low-degree dysplasia may also harbour carcinomas (4). The colonic mucosa of UC patients may also harbour severe molecular abnormalities, such as chromosomal instability (5) and DNA aneuploidy. DNA aneuploidy may be present in dysplastic and non-dysplastic mucosa of UC patients, and is reported to be connected with the duration of disease (6-9). It can be characterized as an independent risk factor for the development of adenocarcinoma in UC (10,11). UC-patients who develop dysplasia or adenocarcinomas are usually considered progressors, whereas patients who do not develop these phenotypes during a lifetime of UC are considered non-progressors. Since DNA-aneuploidy can be considered an independent risk factor for malignancies in UC-colon mucosa, we have also included this as a defining characteristic of a UC-progressor case. The mechanisms behind what makes a UC-colon a progressor or a non-progressor are not fully known, and it is therefore of interest to examine the differences in molecular features of the mucosa between these two types of UC-affected colons. Molecular characteristics of dysplastic as well as non-dysplastic lesions within a progressor that are not found in non-progressors, could be a contribution to the understanding of mechanisms behind carcinogenesis in UC colons, which could serve as a marker for individuals at risk.

Telomerase is a ribonucleoprotein capable of extending the telomeric sequence, generally known to be active in germline cells and inactive in most somatic cells. The two main subunits of telomerase are the catalytic subunit TERT-telomerase

reverse transcriptase (hTERT in humans), and TR (TER), the RNA component (hTR or hTER in humans). In addition to hTERT and hTR, a range of accessory proteins are also closely associated with the complex (12). The level of hTERT is generally assumed to be a limiting factor for assembly of the telomerase complex, and it is reported that hTERT may also play a role in cell proliferation, separate from its role in telomere elongation (13). Telomerase activity is frequently reported in cancer cells, and ~80-90% of all solid tumours, including colorectal cancer, have reported telomerase activity (14-16). Telomerase enables cancerous cells to achieve replicative immortality, which is one of the hallmarks of cancer (17). Telomerase activity may therefore increase the lifespan of a cell, resulting in an accumulation of genetic alterations in the cell that again may contribute to the development of cancer (18). In UC mucosa, however, reports on telomerase activity vary from reduced levels (19), levels not differing from non-UC colons (20-22), to reports on elevated activity (23,24). All these investigations used versions of the Telomeric Repeat Amplification Protocol (TRAP)-assay or PCR-ELISA, valid methods for measuring telomerase activity in a sample by using tissue extracts. Due to the often high levels of inflammation in a UC colon, elevated levels of macrophages and neutrophils are present, and tissue extracts from the colonic mucosa of UC patients may therefore comprise these cell types. A TRAP-assay from colonic mucosal cells may therefore not differentiate between telomerase activity in macrophages and leucocytes in the tissue from that of the epithelial cells, making results difficult to interpret. Notably, in a study on telomerase activity in UC colonic mucosa, where mucosal cells had been separated from stromal cells the results showed different levels of activity in the two sets. Levels of telomerase activity were reported as low in dysplastic mucosa, and a correlation between telomerase activity and inflammation was detected. In this report, DNA-status was not included (22). It has also been speculated as to whether elevated levels of telomerase-activity in UC mucosa are a direct result of enhanced cell proliferation in actively inflamed colon tissue (24).

In the present study, we used immunohistochemistry (IHC) to assess hTERT levels in UC material as it provides the advantage of assessing the protein expression in specific cell types within the tissue examined, thus allowing for the exclusion of macrophages and neutrophils that would obfuscate hTERT level data. We assessed hTERT protein levels using IHC in the colonic mucosal cells of a set of progressor and non-progressor colons of patients suffering from longstanding UC, to investigate whether any differences in hTERT expression were related to progressor status, mucosal dysplastic development or to DNA-ploidy status.

Materials and methods

Patients. Thirty patients suffering from longstanding UC were included in this report. All the patients had suffered from UC for >10 years prior to colectomy, and some patients had suffered as long as 30 years. Patients also varied widely in age at the time when symptoms first presented (from 10 to 60 years old). The 10 non-progressor patients included 5 males and 5 females. The progressors included 17 males and 3 females. Use of this material for research purposes received ethical approval from the Regional Ethics Committee, REK S-06062.

UC colectomies: Progressors and non-progressors. The colectomy specimens have previously been described by Burum-Auensen *et al* (25). The colectomies (n=30) were grouped into progressors and non-progressors, revealing 10 non-progressors that presented no dysplastic lesions, and 20 progressors that all presented at least one area of dysplasia/cancer. The majority of cases also presented DNA aneuploidy.

At least eight sites from each colectomy were examined, and within the progressors 83 non-dysplastic areas were identified, 31 areas indefinite for dysplasia, 29 areas with dysplasia and 8 adenocarcinomas. Since our analyses focused on precancerous morphology changes, the adenocarcinomas were excluded. A total of 18 non-dysplastic and 7 dysplastic areas revealed DNA aneuploidy. The progressor lesions are shown in Table I. By definition the non-progressor lesions were diploid and non-dysplastic.

hTERT IHC. Tissue microarrays (TMAs) from eight sites within each colon were made using a Beecher tissue microarrayer as described previously (25). Core size was 0.6 mm. All cores were previously evaluated by an experienced pathologist (OPFC). At least two tissue cores from each mucosal region were sampled. Two tonsillar sections were used as positive controls. Sections (4 μm) were exposed to 0.5% H_2O_2 solution for 10 min, followed by antigen retrieval in the citrate buffer at pH 6.0. Incubation of TMAs with the primary antibody against telomerase [mouse monoclonal ab5181, dilution (1:500); Abcam, Cambridge, UK], was performed for 1 h at room temperature. Staining was performed using a Ventana Nexes machine using Ventana Iview DAB detection kit (Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's instructions. Sections stained with Tris-buffered saline (TBS) instead of primary antibody served as negative staining controls. hTERT protein expression was defined for each sample as the percentage of positive cells out of 1,200 randomly selected mucosal epithelial cells. Only cells with nuclear staining were counted as positive for hTERT-expression. hTERT-staining of a non-UC control sample and progressor lesions with high and low hTERT levels are presented in Fig. 1. The antibody was tested for specificity using several human cancer cell lines, and a single band of 127 kDa was detected (Fig. 2).

We have previously presented the immunostaining for Ki67 for this material, showing significantly elevated levels of Ki67 in UC colons compared to non-UC controls (25).

Statistical analysis. As each patient included in this study contributed with more than one biopsy, we evaluated the levels of protein expression in relation to the morphologic parameters, as well as the association analyses of protein levels for hTERT and Ki67, by using a multilevel model that compensates for patient differences. The linear mixed model (LMM), with restricted maximum likelihood (REML) estimations and a Bonferroni post-hoc test was performed. Tests were performed in PASW® statistics 18 (Chicago, IL, USA). All tests were two-sided and a p-level of 0.05 denoted significance.

Results

TMA evaluation. TMAs do not consistently exhibit full colonic crypts as observed in whole sections, but since we found the

Table I. Summary of lesions in the progressor colectomies (n=20) according to morphology and DNA-ploidy status.

		Colon specimen #																			
		30	70	71	99	132	159	164	169	174	176	177	191	192	199	205	225	1514	1701	1729	1789
Diploid	Non-dysplasia	5	5	2	1	1	3	1	2	7	2	3	5	4	3	6	5	6	3	0	1
	Indefinite dysplasia	0	0	0	0	2	1	2	1	0	2	1	0	1	5	2	1	1	2	2	0
	Dysplasia	0	2	0	3	1	1	2	0	1	0	0	0	1	0	1	2	0	2	6	0
	Adenocarcinoma[a]	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	2	0	0	0	0
Aneuploid	Non-dysplasia	1	0	0	0	0	3	1	1	1	4	2	2	0	0	0	0	1	1	0	1
	Indefinite dysplasia	0	0	0	0	2	0	1	0	0	0	0	0	1	1	0	0	0	0	0	3
	Dysplasia	0	0	0	1	1	1	2	1	0	0	0	1	0	0	0	0	0	0	0	0
	Adenocarcinoma[a]	1	0	1	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	1

[a]Adenocarcinomas were removed from the analyses.

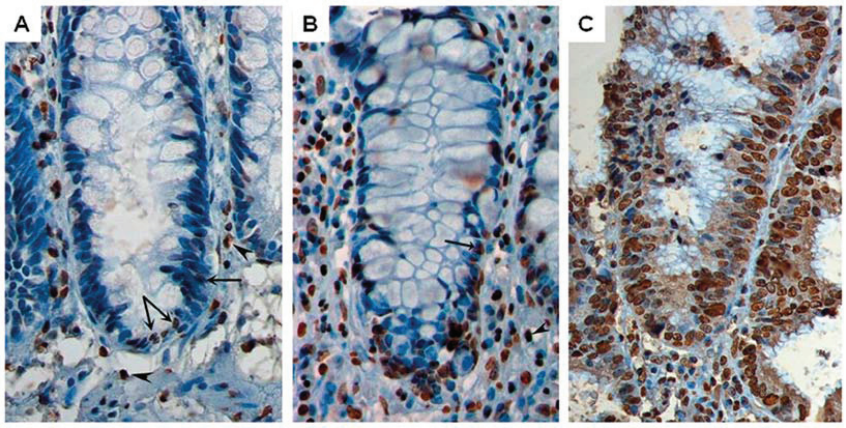


Figure 1. Immunohistochemistry (IHC) for hTERT. (A) Non-UC control sample (from full section), (B) ulcerative colitis (UC)-lesion with low hTERT levels and (C) UC-lesion with high hTERT levels. Images of UC colons are from tissue microarray (TMA)-cores. Arrows mark colonic mucosal cells positive for hTERT in low expression levels, arrowheads mark hTERT-stained leucocytes. Images are x400 magnification.

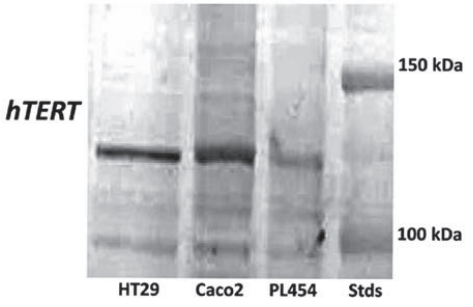


Figure 2. Western blot analysis confirming the specificity of ab5181, a monoclonal antibody for hTERT expression. The antibody was specific, showing a single band at 127 kDa when tested using several cancer cell lines.

expression of hTERT in our study to be evenly distributed throughout the colonic mucosa we concluded that hTERT protein levels could be estimated reliably (data not shown).

As Ki67 protein expression is linked to the growth fraction in UC-colonic mucosa we did not consider TMAs as reliable in assessing Ki67 expression related to dysplastic development.

The assessment of Ki67 protein expression was performed within the same tissue cores as for hTERT protein assessments, thus evaluation of the association between hTERT and Ki67 was considered to be reliable.

Levels of hTERT in the colonic mucosa of progressors vs. non-progressors. Levels of hTERT were significantly elevated ($p<0.001$) in the colonic mucosa of progressors and non-progressors, compared to non-UC controls (Fig. 3). No difference was observed comparing progressor and non-progressor colectomies. Statistically elevated levels of Ki67 in overall UC colons compared to non-UC controls have been previously presented (25).

Levels of hTERT within the colonic mucosa of progressor colectomies. The progressors were divided according to age at onset, as it has been recently shown that progressors with late

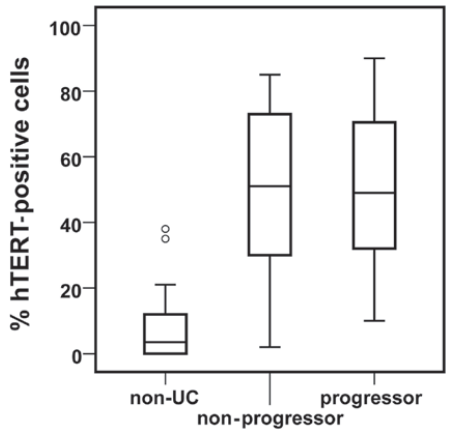


Figure 3. hTERT in ulcerative colitis (UC) progressors, non-progressors and non-UC controls. Protein levels of hTERT detected by immunohistochemistry (IHC) in progressors, non-progressors and non-UC controls.

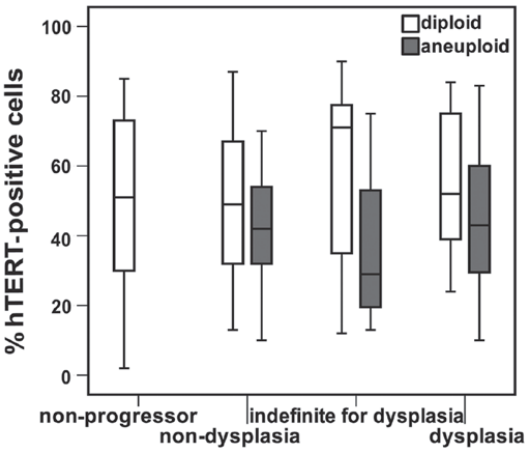


Figure 4. hTERT in diploid and aneuploid lesions of progressors. Expression of hTERT in non-progressors and within areas with different morphologies harbouring diploid or aneuploid populations in progressors.

onset of UC (> 50 years old) differed in telomere biology from progressors with early onset of UC (<50 years old) (26). The results yielded no statistical difference in the protein levels of hTERT when comparing late and early onset UC (p=0.2).

No statistically significant difference in the levels of hTERT expression was detected between diploid lesions and lesions presenting aneuploidy, without correcting for differences in mucosal morphology (p=0.12). No significant differences in hTERT levels were identified between non-dysplastic lesions, lesions indefinite for dysplasia and dysplastic lesions without correcting for DNA-ploidy status (p=0.14). However, when stratifying for mucosal morphology and comparing hTERT protein levels within diploid lesions with those harbouring aneuploid populations, we found that the aneuploid lesions tended to have less hTERT expression than the diploid counterparts (Fig. 4).

Table II. LMM test p-values for hTERT protein levels stratified for DNA-ploidy status within different morphologic stages from progressors.

Morphology	p-value
Non-dysplasia	0.037
Indefinite for dysplasia	0.374
Dysplasia	0.565

LMM, linear mixed model.

Table III. P-values generated from LMM analyses for the association between hTERT and Ki67 protein expression in UC-morphology.

Morphology	p-value
Non-progressor	0.047
Non-dysplasia	0.097
Indefinite for dysplasia	0.102
Dysplasia	0.731

LMM, linear mixed model; UC, ulcerative colitis.

Within the non-dysplastic aneuploid and diploid lesions of the progressor colons the hTERT levels differed to a statistically significant extent (p=0.037) when using LMM accounting for the differences between the patients. The p-values detected using LMM for DNA-ploidy status within each morphologic group are presented in Table II. By ignoring patient differences, and using a t-test, a statistically significant difference was found between hTERT levels stratified for DNA-ploidy status within the lesions scored as indefinite for dysplasia. Diploid lesions had higher levels of hTERT than aneuploid lesions.

Associations between protein levels of hTERT and Ki67 in the colonic mucosa of progressors and non-progressors. An association analysis between hTERT and Ki67 revealed statistically significant results within the non-progressors (p=0.047) when using LMM, compensating for patient variation. No association was detected within the different lesions of the progressors (Table III). No association was detected between the protein levels of hTERT and Ki67 within the progressors when stratifying for DNA-ploidy status.

All analyses were also performed excluding all cases harbouring adenocarcinomas. This did not alter the results to any statistically significant degree.

Discussion

In the present study, we found significantly raised levels of hTERT protein in the mucosa of both progressor and non-progressor UC colectomies compared to non-UC control samples (p<0.001), but no significant difference was detected between hTERT levels in the progressor and non-progressor

colectomies. UC is reportedly a disease of accelerated aging of colonic mucosa (27), with an elevated cell division rate as documented by Greco *et al* (28). The fact that we detected similar hTERT levels in progressor and non-progressor colectomies is consistent with those studies, as the patients had suffered from UC for >10 years. Elevated levels of hTERT were found in mildly active UC in the mucosa of patients suffering from UC on average 6 years (29).

For examination of possible differences in the levels of hTERT within the progressors we stratified the areas of the 20 progressor colectomies by morphological characteristics, and compared diploid areas with areas containing aneuploid clones, using LMM. This comparison showed a pattern of lower hTERT expression in aneuploid lesions within areas of similar morphology. Within the non-dysplastic lesions this difference was statistically significant ($p=0.037$). If each lesion was included in the analysis as independent data entries (Student's t-test), we found a significant difference between hTERT levels in diploid and aneuploid lesions indefinite for dysplasia. However, the protein levels of hTERT vary between patients, a fact that potentially affects our statistical findings, creating false positives. Several of the aneuploid lesions of indefinite dysplastic morphology were found within the same colon (Table I), and this could skew our results. We therefore found the p-values yielded by the LMM analysis controlling for patient variations to be valid. The hTERT-protein expression in diploid, non-dysplastic lesions did not differ from the levels found in the non-progressors, which are all non-dysplastic and diploid. This shows that when the confounding factor of differences in mucosal morphology is accounted for, reduced levels of hTERT are linked to DNA aneuploidy and possibly also associated with its development. Increased levels of hTERT may enhance the proliferative activity of the inflamed tissue harbouring increased levels of reactive oxygen species (ROS), and possibly contribute to the development of dysplasia and cancer. It has been demonstrated that UC colons have enhanced cell proliferation (24) and elevated levels of ROS (30). Both these agents are reported to facilitate telomere shortening. Too short or even missing telomeres can induce breakage-fusion-bridge (BFB) cycles, which again can lead to chromosomal instability (5) and DNA aneuploidy (31,32). Elevated levels of BFB were shown in UC-progressor colons, but DNA-ploidy status of the lesions examined was not investigated (31). Activation of telomerase can prevent BFB-cycles by adding telomeric sequences to short telomeres or broken chromosome ends (33). Our results showing less hTERT present in lesions that contain aneuploid cell populations are consistent with these results.

UC progressors have been shown to differ in mean telomere length depending on the patients' age at disease onset, where early onset (<50 years of age when diagnosed) harboured shorter telomeres than those observed in UC progressors with later UC onset (26). All our patients had suffered from active colitis for >10 years at the time of colectomy and all had presented extensive colitis. Only two progressors were diagnosed with UC after the age of 50, and these did not differ in hTERT expression from the patients diagnosed at an earlier age.

It is possible that any differences in hTERT levels of the colonic mucosa between the progressors and non-progressors were levelled out by continuous impairment of the colonic mucosa due to inflammation and regeneration. Telomeres in UC colonic mucosa are reported to shorten more rapidly than in non-UC mucosa (27,31), and activation of telomerase might be a response to this attrition. This could indicate that hTERT expression is not a biomarker for differentiating a progressor colon from a non-progressor colon prior to colectomy.

A study of four colectomies from patients suffering from UC for >20 years revealed a regional correlation between dysplasia and telomerase activity measured by a version of the ELISA. One patient did not present dysplasia or telomerase activity (23). However, the ELISA method used in the study detected assembled telomerase enzyme complexes, and it was suggested that lack of detected telomerase activity in some of the samples could be due to degradation of the RNA component of the holoenzyme prior to sampling (23). IHC of hTERT may omit tissue-based problems such as partial degradation, as it is based on visual examination of stained formalin or alcohol-fixed, paraffin-embedded tissue.

IHC facilitates the investigation of protein expression in specific cell types within a tissue. This feature can prove valuable when examining UC colons, where a high percentage of leucocytes are generally present in the mucosa. Examining hTERT protein expression by IHC allowed us to assess the differences in the extent of hTERT expression in the colonic mucosal cells, without the confounding contributions from mucosal leukocytes. In a report examining coronary plaques, neutrophils were found to have elevated levels of telomerase activity (34). This is confirmed in our study by the presence of hTERT-positive leucocytes in the lamina propria (Fig. 1).

However, immunohistochemical detection of hTERT has proven to be a difficult task, as some antibodies can also bind to other proteins not associated with telomerase activity (35), antibodies that are not commercially available, or those that are commercially available but have not been proven to be specific (i.e., non-specific cytoplasmic rather than specific nuclear staining). As new antibodies binding to hTERT have become available and tested for binding specificity, reports of hTERT-expression have emerged. Elevated levels of hTERT in precancerous lesions have been identified in gastric tissue (36), and colonic adenocarcinomas (37). The hTERT protein levels of colonic mucosa may provide insight into the transition from normal-looking mucosal morphology towards a possible colorectal cancer, as normal colonic mucosa has low hTERT levels, whereas colorectal cancers have high levels of hTERT (38). In our study the nuclear hTERT staining was very distinct. The monoclonal hTERT antibody used was specific, as confirmed by western blotting of several human cancer cell lines that showed a single band at 127 kDa as expected (Fig. 2). We have previously shown, that progressors harboured significantly more ultra-short telomeres compared to non-progressor colons, and that the difference remained statistically significant when we compared the diploid, non-dysplastic progressor lesions to the non-progressors. In terms of mean telomere length, no difference was found between progressors and non-progressors (39). Thus, an association between mean telomere length and hTERT protein levels seems to exist, whereas no association was observed between the amount of ultra-short telomeres and levels of hTERT in longstanding UC.

In a previous study, our group showed that the proliferation marker Ki67 was significantly elevated in UC colons compared to non-UC control samples, thus confirming that proliferation is enhanced in UC colonic mucosa (25). Also, protein expression of Ki67 has been shown to increase with advancing degree of growth fraction due to the developing stage of dysplasia in the colonic mucosa of the UC colon (40). We found that hTERT expression was significantly associated with the expression of Ki67 within the non-progressor lesions. Within the progressors this association was lost, even when diploid, non-dysplastic lesions were examined separately. Together with our findings of a borderline significant p-value for association between hTERT and Ki67 within progressor non-dysplasia, and no significance detected within the increasing levels of distorted morphology (Table III), it seems the association between proliferation and hTERT protein expression is lost during the development of dysplasia. The lack of difference in hTERT protein levels between progressors and non-progressors, together with elevated amounts of ultra-short telomeres identified in the progressor lesions and the hTERT/Ki67association found only within non-progressors leads to the hypothesis that the positive association between hTERT and Ki67 in the non-progressors may be a protective agent against shortening of the cells telomeres.

In conclusion, we have shown that the protein levels of hTERT were significantly elevated in the mucosa of progressors and non-progressor UC colons compared to non-UC control samples. In the progressor colons, aneuploid non-dysplastic lesions had a significantly lower expression of hTERT than the diploid non-dysplastic lesions, and diploid, non-dysplastic lesions did not differ from the non-progressors with regard to expression of hTERT protein in the colonic mucosal cells, thus low levels of hTERT associated with aneuploidy. We also found that within the non-progressors there was an association of hTERT expression and expression of the proliferation marker Ki67. No association of hTERT/Ki67 protein expression was detected in the progressors, even when only diploid non-dysplastic lesions were examined, indicating that the association of the two proteins may act as a protective mechanism against the development of progressor characteristics within a UC colon.

Acknowledgements

The authors would like to thank Thu Hong Thy Nguyen for helping with western blotting. This study was made possible by the generous funding from South-Eastern Norway Regional Health Authority and by Stiftelsen UNI. These organisations had no role in collecting, analysing or interpreting the data or in writing the report.

References

1. Macdougall IP: The Cancer risk in ulcerative colitis. Lancet 2: 655-658, 1964.
2. Eaden JA, Abrams KR and Mayberry JF: The risk of colorectal cancer in ulcerative colitis: a meta-analysis. Gut 48: 526-535, 2001.
3. Mathy C, Schneider K, Chen YY, Varma M, Terdiman JP and Mahadevan U: Gross versus microscopic pancolitis and the occurrence of neoplasia in ulcerative colitis. Inflamm Bowel Dis 9: 351-355, 2003.
4. Gorfine SR, Bauer JJ, Harris MT and Kreel I: Dysplasia complicating chronic ulcerative colitis: is immediate colectomy warranted? Dis Colon Rectum 43: 1575-1581, 2000.
5. Stenoien DL, Sen S, Mancini MA and Brinkley BR: Dynamic association of a tumor amplified kinase, Aurora-A, with the centrosome and mitotic spindle. Cell Motil Cytoskeleton 55: 134-146, 2003.
6. Hammarberg C, Slezak P and Tribukait B: Early detection of malignancy in ulcerative colitis. A flow-cytometric DNA study. Cancer 53: 291-295, 1984.
7. Meyer KF, Nause SL, Freitag-Wolf S, *et al*: Aneuploidy characterizes adjacent non-malignant mucosa of ulcerative colitis-associated but not sporadic colorectal carcinomas: a matched-pair analysis. Scand J Gastroenterol 48: 679-687, 2013.
8. Fozard JB, Quirke P, Dixon MF, Giles GR and Bird CC: DNA aneuploidy in ulcerative colitis. Gut 27: 1414-1418, 1986.
9. Meling GI, Clausen OP, Bergan A, Schjølberg A and Rognum TO: Flow cytometric DNA ploidy pattern in dysplastic mucosa, and in primary and metastatic carcinomas in patients with longstanding ulcerative colitis. Br J Cancer 64: 339-344, 1991.
10. Gerling M, Nousiainen K, Hautaniemi S, *et al*: Aneuploidy-associated gene expression signatures characterize malignant transformation in ulcerative colitis. Inflamm Bowel Dis 19: 691-703, 2013.
11. Gerling M, Meyer KF, Fuchs K, *et al*: High frequency of aneuploidy defines ulcerative colitis-associated carcinomas: a comparative prognostic study to sporadic colorectal carcinomas. Ann Surg Jun: 4, 2010 (Epub ahead of print).
12. Sauerwald A, Sandin S, Cristofari G, Scheres SH, Lingner J and Rhodes D: Structure of active dimeric human telomerase. Nat Struct Mol Biol 20: 454-460, 2013.
13. Mukherjee S, Firpo EJ, Wang Y and Roberts JM: Separation of telomerase functions by reverse genetics. Proc Natl Acad Sci USA 108: E1363-E1371, 2011.
14. Kim NW, Piatyszek MA, Prowse KR, *et al*: Specific association of human telomerase activity with immortal cells and cancer. Science 266: 2011-2015, 1994.
15. Chadeneau C, Hay K, Hirte HW, Gallinger S and Bacchetti S: Telomerase activity associated with acquisition of malignancy in human colorectal cancer. Cancer Res 55: 2533-2536, 1995.
16. Shay JW and Bacchetti S: A survey of telomerase activity in human cancer. Eur J Cancer 33: 787-791, 1997.
17. Hanahan D and Weinberg RA: Hallmarks of cancer: the next generation. Cell 144: 646-674, 2011.
18. Baykal A, Rosen D, Zhou C, Liu J and Sahin AA: Telomerase in breast cancer: a critical evaluation. Adv Anat Pathol 11: 262-268, 2004.
19. Usselmann B, Newbold M, Morris AG and Nwokolo CU: Deficiency of colonic telomerase in ulcerative colitis. Am J Gastroenterol 96: 1106-1112, 2001.
20. Engelhardt M, Drullinsky P, Guillem J and Moore MA: Telomerase and telomere length in the development and progression of premalignant lesions to colorectal cancer. Clin Cancer Res 3: 1931-1941, 1997.
21. Kleideiter E, Friedrich U, Möhring A, *et al*: Telomerase activity in chronic inflammatory bowel disease. Dig Dis Sci 48: 2328-2332, 2003.
22. Risques RA, Lai LA, Himmetoglu C, *et al*: Ulcerative colitis-associated colorectal cancer arises in a field of short telomeres, senescence, and inflammation. Cancer Res 71: 1669-1679, 2011.
23. Holzmann K, Klump B, Weis-Klemm M, *et al*: Telomerase activity in long-standing ulcerative colitis. Anticancer Res 20: 3951-3955, 2000.
24. Myung SJ, Yang SK, Chang HS, *et al*: Clinical usefulness of telomerase for the detection of colon cancer in ulcerative colitis patients. J Gastroenterol Hepatol 20: 1578-1583, 2005.
25. Burum-Auensen E, De Angelis PM, Schjølberg AR, Røislien J, Andersen SN and Clausen OP: Spindle proteins Aurora A and BUB1B, but not Mad2, are aberrantly expressed in dysplastic mucosa of patients with longstanding ulcerative colitis. J Clin Pathol 60: 1403-1408, 2007.
26. Salk JJ, Bansal A, Lai LA, *et al*: Clonal expansions and short telomeres are associated with neoplasia in early-onset, but not late-onset, ulcerative colitis. Inflamm Bowel Dis 19: 2593-2602, 2013.
27. Risques RA, Lai LA, Brentnall TA, *et al*: Ulcerative colitis is a disease of accelerated colon aging: evidence from telomere attrition and DNA damage. Gastroenterology 135: 410-418, 2008.

28. Greco V, Lauro G, Fabbrini A and Torsoli A: Histochemistry of the colonic epithelial mucins in normal subjects and in patients with ulcerative colitis. A qualitative and histophotometric investigation. Gut 8: 491-496, 1967.
29. Sipos F, Galamb O, Herszényi L, *et al*: Elevated insulin-like growth factor 1 receptor, hepatocyte growth factor receptor and telomerase protein expression in mild ulcerative colitis. Scand J Gastroenterol 43: 289-298, 2008.
30. Roessner A, Kuester D, Malfertheiner P and Schneider-Stock R: Oxidative stress in ulcerative colitis-associated carcinogenesis. Pathol Res Pract 204: 511-524, 2008.
31. O'Sullivan JN, Bronner MP, Brentnall TA, *et al*: Chromosomal instability in ulcerative colitis is related to telomere shortening. Nat Genet 32: 280-284, 2002.
32. Lo AW, Sabatier L, Fouladi B, Pottier L, Ricoul M and Murnane JP: DNA amplification by breakage/fusion/bridge cycles initiated by spontaneous telomere loss in a human cancer cell line. Neoplasia 4: 531-538, 2002.
33. Hackett JA and Greider CW: Balancing instability: dual roles for telomerase and telomere dysfunction in tumorigenesis. Oncogene 21: 619-626, 2002.
34. Narducci ML, Grasselli A, Biasucci LM, *et al*: High telomerase activity in neutrophils from unstable coronary plaques. J Am Coll Cardiol 50: 2369-2374, 2007.
35. Wu YL, Dudognon C, Nguyen E, *et al*: Immunodetection of human telomerase reverse-transcriptase (hTERT) re-appraised: nucleolin and telomerase cross paths. J Cell Sci 119: 2797-2806, 2006.
36. Duarte MC, Babeto E, Leite KRM, *et al*: Expression of TERT in precancerous gastric lesions compared to gastric cancer. Braz J Med Biol Res 44: 100-104, 2011.
37. Simsek BC, Pehlivan S and Karaoglu A: Human telomerase reverse transcriptase expression in colorectal tumors: correlations with immunohistochemical expression and clinicopathologic features. Ann Diagn Pathol 14: 413-417, 2010.
38. Hiyama E, Hiyama K, Yokoyama T and Shay JW: Immunohistochemical detection of telomerase (hTERT) protein in human cancer tissues and a subset of cells in normal tissues. Neoplasia 3: 17-26, 2001.
39. Friis-Ottessen M, Bendix L, Kølvraa S, Norheim-Andersen S, De Angelis PM and Clausen OP: Telomere shortening correlates to dysplasia but not to DNA aneuploidy in longstanding ulcerative colitis. BMC Gastroenterol 14: 8, 2014.
40. Andersen SN, Rognum TO, Bakka A and Clausen OP: Ki-67: a useful marker for the evaluation of dysplasia in ulcerative colitis. Mol Pathol 51: 327-332, 1998.