Dissertation

Cancer stem cell gene variants and their prognostic value in adjuvant setting of colon cancer

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submitted by

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the „Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz.

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Disclosures

This doctoral thesis was the basis for the elaboration of a manuscript, which has been published in Anticancer Research. The published manuscript *"Cancer Stem Cell Gene Variants in CD44 Predict Outcome in Stage II and Stage III Colon Cancer Patients."(1)* was drafted by the doctoral candidate, Michael Stotz. Therefore, significant parts of the doctoral thesis are similar to the published manuscript (with permission of Anticancer research).

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List of abbreviations (in alphabetical order)

AJCC	American Joint Committee on Cancer
CD	cluster of differentiation
CDC	Centers for Disease Control and Prevention
CEA	carcinoembryonic antigen
CI	confidence interval
CIMP	CpG island methylator phenotype
CRC	colorectal cancer
CSC	cancer stem cell
DFS	disease-free survival
DPP4	Dipeptidylpeptidase 4
5-FU	5-fluorouracil
EGFR	epidermal growth factor receptor
IGF-1	Insulin-like Growth Factor Typ 1
IRB	Institutional Review Board
LV	leucovorin
HR	hazard ratio
MMR	mismatch repair
MSI	microsatellite instability
MSI-H	high-frequency microsatellite instability
MSI-L	low-frequency microsatellite instability
MSS	microsatellite stable
OS	overall survival
PROM1	Prominin-Like Protein 1
Rs	Reference SNP cluster ID
Sd	standard deviation
SNP	single nucleotid polymorphism
TNM	tumor, node, metastases
TTR	time to tumor recurrence

Abstract

Background:

Growing evidence suggests that human cancers are stem cell diseases and recent data support the existence of cancer stem cells (CSCs) in a variety of cancer entities including colon cancer. These CSCs were shown to be capable of initiating tumor development and progression. Several studies have suggested CD133, CD26 and CD44 as markers of tumor-initiating cells of colon cancer. The purpose of the present study was to assess the impact of single nucleotid polymorphisms (SNPs) in stem cell related genes on clinical outcome in a large cohort of colon cancer patients with clinical stage II and III.

Methods:

Data from 599 consecutive patients with colon cancer stage II and III, treated between 1995 and 2011 at a single centre, were evaluated retrospectively. Genomic DNA was extracted from paraffin-embedded normal tissue distant from the tumor to obtain germline DNA. Allelic distribution of polymorphisms was tested for deviation from Hardy–Weinberg equilibrium using χ^2-test. The association of polymorphisms with time to recurrence (TTR) and overall survival (OS) was analyzed using Kaplan–Meier curves and compared by log-rank test. Case-wise deletion for missing polymorphisms was used in univariable and multivariable analyses.

Results:

CD44 rs187115 showed a statistically significant association with TTR – patients carrying at least one G allele had a significant reduced risk of recurrence compared to patients with the homozygous A/A variant (HR 0.67, 95% CI 0.48-0.94, p=0.019). CD44 rs13347 showed a statistically significant association with OS. Patients carrying at least one T allele in rs13347 had a significantly reduced risk of death compared to patients with the homozygous C/C variant (HR 0.61, 95% CI 0.41-0.92, p=0.019). None of the other investigated polymorphisms (CD44 rs187116, CD44 rs7116432, CD44 rs353639, DPP4 rs2268889, DPP4 rs3788979, DPP4 rs7608798 and CD133 rs2240688) was associated with either TTR or OS.

Conclusion:

Our data show that the germline variants rs13347 and rs187115 in the stem cell gene CD44 are prognostically relevant in stage II and III colon cancer patients (1).

Zusammenfassung

Hintergrund:

Es lässt sich zunehmend beweisen, dass Krebserkrankungen beim Menschen Stammzellenerkrankungen sind und aktuelle Daten belegen das Vorhandensein von Krebsstammzellen (CSCs) bei einer Vielfalt von Tumorentitäten, so auch bei Kolonkarzinomen. Es konnte dargestellt werden, dass diese CSCs die Tumorentwicklung auslösen und auch für die Tumorprogredienz verantwortlich sind. Mehrere Studien haben gezeigt, dass CD133, CD26 und CD44 Marker für tumorinitiierende Zellen bei Kolonkarzinomen sind. Ziel dieser Studie war es, den Einfluss von Single-Nucleotid-Polymorphismen (SNPs) auf Stammzellen bezogenen Genen bezüglich Behandlungsergebnissen in einer großen Kohorte von PatientInnen mit Kolonkarzinomen in den Stadien II und III zu bewerten.

Methoden:

Die Daten von 599 fortlaufenden PatientInnen mit Kolonkarzinomen der Stadien II und III, die zwischen 1995 und 2011 an einem einzigen onkologischen Kompetenzzentrum behandelt wurden, wurden retrospektiv evaluiert. Die genomische DNA wurde aus Paraffin-eingebettetem normalem Gewebe, weit entfernt vom Tumor extrahiert, um die Keimbahn-DNA zu erhalten. Die allelische Verteilung wurde auf Abweichung vom Hardy-Weinberg Gleichgewicht unter Verwendung des x^2 Tests getestet. Der Zusammenhang von Polymorphismen mit TTR und OS wurde mittels Kaplan-Meier Kurven analysiert und durch log-rank Test verglichen. Fallweise Löschung aufgrund fehlender Polymorphismen wurde in univariablen und multivariablen Analysen vorgenommen.

Resultate:

CD44 rs187115 zeigte einen statistisch gesehen signifikanten Zusammenhang mit TTR – PatientInnen, die mindestens ein G Allel trugen, hatten ein signifikant geringeres Rezidivrisiko verglichen mit PatientInnen mit der homozygoten A/A Variante (HR 0.67, 95% CI 0.48-0.94, p=0.019). CD44 rs13347 zeigte einen statistisch signifikanten Zusammenhang mit OS. PatientInnen, die mindestens ein T Allel auf rs13347 trugen, hatten ein signifikant geringeres Sterberisiko verglichen mit PatientInnen mit der homozygoten C/C Variante. (HR

0.61, 95% CI 0.41-0.92, p= 0.019). Keiner der übrigen untersuchten Polymorphismen (CD44 rs187116, CD44 rs7116432, CD44 rs353639, DPP4 rs 2268889, DPP4 rs3788979, DPP4 rs7608798 und CD133 rs2240688) konnte mit TTR oder OS in Zusammenhang gebracht werden.

Ergebnis:

Unsere Daten zeigen, dass die Keimbahnvarianten rs13347 und rs187115 auf dem Stammzellgen CD44 bei Kolonkarzinom-PatientInnen der Stadien II und III prognostisch relevant sind.

Background

Colon cancer is one of the most common cancer entities in the Western World. Treatment as well as diagnostic improvements have significantly improved survival rates in the last decades. Chemotherapy remains the backbone of treatment in curative as well as palliative situation. In stage III (lymph nodes positive) and stage IV (distant metastasis) the role of chemotherapy is well investigated and its application is usually recommended. However, in stage II disease the role of chemotherapy is not fully clear. Moreover, there is a fundamental lack in comprehension of inter-individual response rates to the same chemotherapy courses in stage II as well as stage III patients. Therefore, recently biomarker research has become more and more dominant to meet each patient's needs for individualized treatment approach. This is not just a phenomenon in colon cancer – in nearly all tumor entities the individual tumor behavior becomes more and more clear. Many different fields of research like e.g. immunological and chemical blood parameters, genetic as well as protein variations, microRNA and their role in tumor behavior as well as tumor stem cells are currently strongly investigated. Findings of this research have already been implemented in clinical practice – in metastatic colon cancer e.g. the RAS mutation test and its consequences for the application of EGFR therapy or in lung cancer the testing of mutations in the genes ALK, ROS1 or EGFR and ensuing treatment regimes. However, despite great advances in the treatment of colon cancer the problem of chemoresistance in the course of tumor disease and progress hasn't been solved yet. The theory, that so-called tumor stem cells on the one hand and selection pressure by chemotherapy on the other hand might be an explanation for this problem.

Dick and Bonnet first identified a subpopulation of cells in AML (acute myeloid leukemia) in 1997, thereby laying the foundation for intensive research activities dealing with cancer stem cells (CSC) in the last two decades (2). They demonstrated, that cells were capable of initiating human AML in non-obese diabetic mice with severe combined immunodeficiency disease. These cells hold the ability of differentiation and proliferation as well as the aptitude for self-renewal. It is estimated that 0.1 to 20 percent of the tumor tissue is composed of cell populations that possess the capability for tumor promotion, initiation and differation into a variety of tumor cells lines (3). Moreover, these cells are more resistant to therapeutic strategies like chemotherapy or radiation, which explains progression after initially achieved remission (4). Germline polymorphisms` role on chemoresistance and cancer outcome is a mounting research field (5-7). Germline genetic variability within the genes is frequent, including multiple single-nucleotide polymorphisms (SNPs). These DNA-sequence variations

may lead to modified gene function and/or activity, which may explain inter-individual differences in patient's tumor outcome (8, 9).

Introduction

Colorectal cancer ranks third place of cancer overall and third leading cause of cancer related death affecting both, males and females (10). The incidence in Austria decreased from 36.9 cases per 100 000 humans (men and women) in 1992 to 28.5 in 2012. In the same time period the mortality also has fallen from 20.4 to 11.3 cases per 100 000 humans (11). This matches approximately with the CDC's incidence for CRC, which was 40 in 2011 (12). These enhancements in mortality and incidence are probably results of earlier diagnosis through screening procedures as well as effective cancer prevention programs and of course modern treatment modalities. Paradoxically, the contrary is valid for patients younger than fifty, suffering from CRC, as in this group the incidence shows an increasing curve. Up to now no satisfactory explanation for this trend has been provided (13).

There are several well-known risk factors for CRC development like the appearance of CRC in a first-degree relative, high-risk adenomas, inflammatory bowel diseases and genetic predisposition, including hereditary nonpolyposis coli and familial adenomatous polyposis (14-16). Genetically determined colon cancer predisposition is rare in total, e.g. the Lynch syndrome, as the most common form, accounts for about 2 to 4 percent of all colorectal cancer cases (17). However, it is an excellent example for the role of germline mutations and the potent functional consequences of genetic aberrations in DNA mismatch repair (MMR) genes (e.g. MLH1, MSH2, MSH6, and PMS2). Nonetheless, loss of expression of MMR genes is also found in about 10 to 15 percent of sporadic CRCs (18).

Lifestyle risk factors also seem to correlate with colorectal incidence, e.g. physical activity, nutritional behavior, smoking and alcohol consumption. There are large regional differences in CRCs incidence, which seem to be caused mainly by environmental and nutrition facts. Genetic factors apparently seem to play a minor role. This can be observed in individuals immigrated from low to high-risk countries and vice versa. Underlying pathophysiological processes might be obesity usually caused by high calorie food intake which causes high levels of IGF-1 – a potential stimulator of intestinal mucosal changes. To what extend the physical activity is a significant protective factor for CRC development remains unclear as overweight and low physical activity are strongly correlated. On the other hand, smoking and a high alcohol intake are more likely risk factors for CRC development according to e.g Reid et al. as well as Giovannucci et al. (19-22). Other possible risk or protective factors like

calcium or folid acid intake as well as acetylsalicylic acid or hormone replacement therapy are still under discussion and given data is conflicting (23-25). As already mentioned chronic inflammatory bowel diseases are predisposing for the development of colorectal cancer – the risk is correlating with time, which means that about ten years after onset of the inflammatory disease the risk of CRC increases about one percent per year. This is another excellent example of how chronic inflammation promotes tissue damage and consequently the development of genetic aberrations.

There are several genetic disorders, which lead to an elevated risk of CRC development, like familial adenomatous polyposis (FAP), the hereditary non-polyposis CRC (Lynch-Syndrome; HNPCC), the familial juvenile polyposis, the Peutz-Jeghers-Syndrom, the Cowden-Syndrome and the hereditary mixed polyposis syndrome. FAP occurs equally in females and males with an incidence of 1.3 cases per 1 000 000 individuals. About one third of FAP is caused by sporadic mutations of the APC-gene, whereas nearly two third of FAP are inherited autosomal dominant. The gene is located on chromosome 5q21-5q22. and nearly 1 000 different germline mutations have been discovered, being able to damage the APC coded protein (26, 27). FAP goes along with usually more than 100 adenomas in the gastrointestinal tract and is associated with an approximately 100 percent risk of CRC development after about 30 years.

Hereditary non-polyposis colorectal cancer (HNPCC) is transmitted autosomal dominant and the genetic cause of HNPCC is related to DNA repair dysfunction. Most cases (nearly 90 percent) are caused from a mutation in one allele of one of the DNA mismatch repair genes - MLH1 and MSH2 (up to 90%), whereas only 10 percent are associated with MSH6 and much more rare with PMS2. This loss of function in MMR causes defects in DNA repair, thereby leading to high DNA microsatellite instability, which has recently become more and more important in oncological treatment decisions (27).

Boland et al. were the first who validated a panel of five microsatellites and characterized tumors on the basis of high frequency MSI, if two or more of the five markers show instability (like insertion or deletion mutation) and low frequency MSI, if only one of the five markers shows instability. The group found that the clinical and pathological phenotype in MSI-H tumors is different to MSI-L and MSS tumors. MSI-H colorectal tumors are associated with a prognostically better clinical course, have a greater mucinous component, contain lymphocytic infiltration, are more often poorly differentiated and more frequently occur in the proximal colon than MSI-L or MSS tumors (28). Up to now there is no commonly accepted explanation for the clinically worse course of MSI-L patients, although it

could be proven in several studies (29, 30). However, especially the lymphocytic infiltration is of special interest, as already in 2008 Schwitalle et al. showed that HNPCC-associated CRC frequently go along with a potent immune response, resulting from the MSI-induced generation of novel tumor-specific carboxy-terminal frameshift peptides (FSPs) (31). More recently Alvi et al. investigated signet ring cell colorectal cancer patients and their mutation status focusing on MSI, CpG island methylator phenotype (CIMP) and BRAF V600E mutation. Signet ring cell colorectal cancers are generally rare (0.1-2.4% of all CRC cases) but usually associated with high malignancy and a poor differentiation consequently leading to a presentation at an advanced stage with a poor five-year survival rate of less than 20 percent. The group of Alvi et al. observed a significant higher infiltration of CD3+ T-lymphocytes in MSI cases compared to MSS patients as well as a higher expression of PDL1 in MSI cases compared to MSS (32). These findings support recent recent findings highlighting the role of immune checkpoint inhibitor therapies in MSI tumors (33).

Colon cancer is staged according to the TNM (tumor, node, metastases) system – defined through the AJCC Staging Manual. (8_{th} edition released 2016) For a long period of time the TNM system was the only relevant basis of decision-making. However, in the more recent past it has been extended by pathologic factors like perineural-, vascular or lymphovascular invasion and the radial resected margin status (34). Colorectal carcinomas are rather located in the right colon in younger individuals and mostly in the area of rectosigmoid in people older than forty. Colorectal carcinomas can show exophytic, endophytic, diffusely infiltrative, ulcerative growth or even grow circumferentially in the colorectal wall with frequent overlaps of these growing types. CRCs like most other tumor entities are graded into well, moderately, poorly and undifferentiated lesions (G1-4), which in fact is dependent of the extent of glandular appearances (35).

Survival rates in colon cancer are highly stage dependent with 5-year survival rates from 90 percent (stage I), decreasing to 70 percent (stage II and III) and about 13 percent in case of distant metastasis (36). Tumor recurrence after curative surgery still remains a major problem. 5-fluorouracil based chemotherapy is the standard treatment for patients with stage III and high-risk stage II colon cancer after curative surgery to significantly reduce the risk of relapse (37). Thus, in non-metastatic disease 5-year survival rates range from about 40 to 90 percent, depending on the clinical stage (38). A large number of colon cancer patients does not benefit from adjuvant cytotoxic treatment because they develop tumor recurrence or distant metastases despite adjuvant treatment. Therefore, more precise biomarkers are needed to

guide adjuvant treatment through patient classification in order to avoid unnecessary chemotherapy and increase outcome of colon cancer patients (39, 40).

The majority of colorectal cancers develop on the so-called adenoma-carcinoma sequence, which is a multiple steps process from a healthy mucosa to adenomas and dysplasia to ultimately an invasive cancer disease (41). This multi-level process is triggered by DNA damages like hypermethylation of the DNA, loss of DNA on APC or DCC gene on chromosomes 5 and 18 respectively and point mutations in the KRAS protooncogene. Moreover, the tumor suppressor gene p53 is functionally harmed by the loss of the allele on chromosome 17(42, 43).

The molecular or genetic aberrations which initiate colon cancer development and progression are manifold and seem to start with a dysfunction of APC suppressor gene hence leading to dysregulation of interaction with E-cadherine and gene transcription. Cellular responses to growth signals are mediated by the intracellular RAS/RAF/MEK/mitogen-activated protein kinase (MAPK) cascade, which KRAS and BRAF belong to. KRAS mutations occur frequently (up to 30–50 percent) in CRCs whereas BRAF mutations are clearly less frequent (about 10 percent) (44). However, mutations and consequently activation in proto-oncogenes like ras or c-myc or inactivation of tumor suppressor genes lead to tumor progression or to be more precise to development of malign cells out of dysplastic ones. Another important step in tumor course clearly is the inactivation of the p53 gene on chromosome 17 which leads to dysregulation of the cyclin-dependent kinase inhibitor which complexes with proliferating cell nuclear antigen and genes resulting in apoptosis (45-47). In CRC the most common type of genomic instability is chromosomal which leads to APC, p53 or SMAD inactivation (48, 49). On the other hand in CRC genes are often inactivated by silencing of genes which is commonly mediated by aberrant DNA methylation (50). In the CRC genome, in contrast to normal cells, cytosine methylation is modest global depleted. Moreover, in CRC cells there is also a considerable acquisition of aberrant methylation within certain promoter-associated CpG islands, which can induce epigenetic silencing of gene expression. Among the spots that are subject to aberrant methylation, a subgroup is apparently aberrantly methylated as a group. This phenomenon is called CpG island methylator phenotype (CIMP, or CIMP-high). It is seen in about 15% of CRCs, being present in nearly all such tumors with aberrant methylation of MLH1. A third example of aberrant methylation is exemplified by exon 1 of the gene encoding vimentin. Even though this spot cannot be observed in normal colon mucosa or CRC, it is aberrantly methylated in 53 to 83% of patients

with CRC in a way that is independent of CIMP (51). Thus, various genetic factors seem to contribute to the development and clinical course of colorectal cancer and it is now widely accepted that multiple factors contribute to the efficacy of chemotherapy and that treatment should be optimized on an individual case-specific basis.

Genes

DPP4/CD26

DPP4 is a transmembrane glycoprotein with proteolytic activity and several regulatory peptides are split by this enzyme (52). Consequently, DPP4 could be shown to be involved in cancer related processes, such as migration, apoptosis, invasion and sensitivity to chemotherapy (53). DPP4 is not only enzymatically active but also interacts with extracellular matrix proteins like collagen or fibronectin. It further functions as main binding protein for the ecto-adenosine deaminase and plays a role in immune modulation by T-cell activation and participation in several signaling pathways (54-57). The enzymatically active soluble form of DPP4 (sDPP4) has been described in several biological fluids including the serum of patients (52, 58). Consequently sDDP4 has been investigated in several malignancies for its biomarker potential, including colon cancer (59-63). In colorectal cancer the level of sDDP4/CD26 was significantly associated with tumor stage since patients with metastatic disease had higher levels than individuals with localized disease, who in turn had higher levels than healthy individuals (61).

However, existing data about its beneficial or harmful role in cancer development or progression is conflicting (64-66). Apparently DPP4, as a regulator of several biological pathways, functions as tumor suppressor as well as a tumor promoter, depending on cancer cell types. Tumor suppressor properties could be shown in lung cancer, prostate cancer, melanoma and neuronal tumors (67-71). In Ewing sarcoma, mesothelioma, colorectal cancer and hematological malignancies the findings were diametrically opposite (72-76). Liang et al. recently showed that a knockdown of DPP4 resulted in a decreased cell growth in urothelial carcinoma. Moreover, after DPP4 knockdown, the migratory as well as invasion ability of tumor cells were suppressed. Clinically, a DPP4 overexpression was associated with poor prognostic markers, like nodal, lymphovascular permeation, perineural invasion and distal metastasis (75). In colorectal cancer DPP4 was identified as a CSC marker co-expressed on CD133+ cancer cells (77). Pang et al. observed, that the presence of DPP4/CD26(+) cells in primary tumors predicted distant metastasis on follow-up and was associated with chemoresistance and enhanced invasiveness. A downregulation of DPP4/CD26 led to decreased cell aggressiveness (lower rate of migration, invasion and adhesion potential). The group also proved that the treatment of cells with oxaliplatin and 5-FU accumulated the CD133+CD26+ CSC population by failing to eliminate this CSC subpopulation, hereby favouring distant metastasis (74). In another mouse model, the inhibition of DPP4/CD26 with

vildagliptin suppressed both, the incidence and growth of lung metastases, thereby supporting the findings of Pang et al. (78). Lam et al. as well as Lieto et al. found that an increased expression of DDP4 goes along with a higher tumor stage and a lower survival respectively higher recurrence rate (79, 80).

PROM1/CD133

CD133 is a trans-membrane cell-surface glycoprotein and can be found in several normal cell types, like neuronal, endothelial and hematopoietic progenitor cells (81). It was first described in neuroepithelial mice stem cells and afterwards in human cells (82). CD133 seems to be involved in epidermal mesenchymal coaction, cell differentiation and proliferation by influencing the WNT signalling pathway (83-86). However, its' physiologic function is still fairly unknown. A direct interaction between CD133 and the VEGF (vascular endothelial growth factor) was found by the groups of Gehling and Adini. Adini et al. investigated PROM1 function in two cell lines with high expression of prominin-1. When PROM1 was knocked down, capillary formation of endothelial cells was intercepted in vitro and lowered in vivo. In melanoma cells the knockdown resulted in a reduced growth rate in vivo and a reduced interaction potential with the VEGF, which was associated with an increased apoptosis (87). Thus, it is imaginable that CD133 plays a role in physiologic neovascularisation and tumor angiogenesis as well (88). In a recent review pooled data revealed that high CD133 expression does not only correlate with poor prognostic outcome in pancreatic cancer patients but is also associated with tumor grade, tumor size and lymph node metastasis (89). Already in 2007 CD133 was shown to be a marker for CSCs in colorectal cancer. O'Brian et al. showed, that CD133+ cells were able to sustain themselves and establish tumor heterogeneity and further differentiation (90). Ricci-Vitiani et al. investigated the role of tumorigenic cells in colon cancer and found that the high-density CD133+ population, that accounted for about 2.5 percent of the tumor cells, reproduced the tumor in immunodeficient mice. For CD133- cells this result could not be shown. Tumors were serially injected for several generations, with progressively faster tumor growth in each generation (91).

The studies of Ricci-Vitiani and O'Brian suggest that CD133+ cells have a highly increased capacity for initiating tumors than CD133- cells. In stage II colon cancer patients CD133 expression was significantly associated with preoperative serum CEA level ($p = 0.006$) and tumor grading ($p = 0.019$). An elevated expression was a significant predictor for poor DFS and OS in multivariate analysis ($p = 0.026$) (92). Similar results in colon cancer were found in several studies (93-96). Almost all colorectal cancers arise from adenomas, according to the adenoma carcinoma sequence model (97). Nonetheless, CD133's role in this sequence remains unclear. Recently the CD133 expression could be shown to be associated with differentiation status ($p = 0.003$) and tumor size in adenomas (98). Moreover, the CD133 mRNA expression in tumors was found to correlate with a lower survival rate (99, 100) as an

elevation of CD133 mRNA goes along with more frequent metastasis formation in rectal cancer (101). Similar findings were found by Horst et al. as well as Li et al. (102, 103). On the other side Choi et al. could not show an association of CD133 expression and survival (104). Taken together, high CD133 expression seems to be associated with poor survival in several tumor entities including colon cancer (105). Moreover, CD133 positive subpopulations of colon cancer cells showed higher tumorigenic potential in several studies and were related to chemoresistance, resistance to radiation and an elevated metastatic potential (90, 106-109).

CD44

CD44, a major cell adhesion molecule, plays a role in various cellular processes like migration, cellular binding and regulation of growth and homing of lymphocytes (110). There are several splice forms of CD44, which have been shown to correlate with cancer progression (111). Considering CD44 promoting several tumorigenic processes it seems likely that the *CD44* gene harbours functional genetic variants that may have potential as molecular prognostic and/ or predictive markers in colon cancer. Like CD 133, it is also a transmembrane glycoprotein, functioning as a receptor for the extracellular matrix protein hyaluranon. The beta-catenin/Tcf-4 complex is another possible activation pathway for CD44 expression in colon cancer cells (112). In 2002 van de Wetering et al. found this complex as a major turning point that controls proliferation versus differentiation in healthy and malignant intestinal epithelial cells. In the study of Dalerba et al. CD44 was firstly identified as a possible CSC marker. In their study injection of CD44+ cells from human colon cancer cells led to tumor development in forty percent of in NOD/SCID mice whereas in case of CD44- cell injection none of the mice developed a tumor disease (113). Du et al. showed, that siRNA reduction of CD44 in colon cancer cells inhibited tumor formation and colony-forming capability reduced colony when injected into SCID mice. 100 CD44+ cells from a patient's tumor were able to induce a xenograft tumor in vivo. A single CD44+ cell from a colon tumor could form a sphere in vitro, which had characteristic stem cell properties and generated a xenograft tumor with the same properties of the primary tumor. The group also found that in comparison to CD133+ cells, CD44+ cells have greater tumor initiating potential (114). Misra et al. inhibited the expression of CD44v6, which lead to depletion of the colon tumor cells to signal through hyaluronan-CD44v6 interactions. They further demonstrated that inhibition of the CD44v6 expression reduces the signaling through a hyaluronan/CD44v6-pErbB2-Cox-2 interaction pathway and reduce adenoma number and growth (115). Increased CD44 might be an indicator for presence of colon CSCs because a high CD44 expression foregoes p53 and KRAS gene damage and is associated with a higher amount of polyps in the colon (116, 117). To sum up CD44 could be a useful therapeutic target which seems to be more specific as CSC marker than CD133.

Single nucleotide polymorphisms

SNP occur approximately once every 100 to 300 bases. In healthy cells, there is usually a 50 percent ratio of two alleles for any SNP because one allele stems from the mother whereas the other one is driven from the father. Nonetheless, in tumor cell lines the ratio can be shifted due to chromosomal changes (118). Single nucleotide polymorphisms are substitutions of one nucleotide for another. They are the most frequent form of genetic variation and responsible for interindividual differences. Germline DNA sequence variations might modify protein activity or even production leading to different tumor course by affecting chemoresistance or metastatic capacity. Human genome mapping has led to a giant field of research through the ability and the interest in investigating genetic variations and the potential role in cancer disease. However, it is estimated that an individual carries about 2.8 to 3.9 million single-base pair variants (119). Heterozygosity is enormous in genes and locally different and e.g. more frequent in non-coding regions. SNPs are defined as single-base pair change that occurs with a frequency of at least 1 percent in a population. However, it is not the frequency of a SNP but its location within the gene that causes its functional role, which indeed is hard to define. As mentioned above, SNPs occur in the coding as well as the non-coding region in genes. Synonymous SNPs do not influence the protein sequence whereas nonsynonymous SNPs are likely to do so, thereby changing a protein's amino acid, which may result in functional changes (120). Previously, SNPs in non-coding regions have been thought to be unimportant but conversely they indeed can influence several procedures like gene splicing or the sequence of so called non-coding RNA.

Therapeutic approach and decision in adjuvant situation

Stage II

Especially in stage II colon cancer several risk factors have been described to distinguish between patients with low risk of recurrence and patients with a high risk. These are: lymph nodes sampling <12; poorly differentiated tumor; vascular or lymphatic or perineural invasion; tumor presentation with obstruction or tumor perforation and pT4 stage. This is of great clinical importance because patients, who have at least one of these risk factors should be offered to adjuvant chemotherapy (121). Adjuvant chemotherapy in CRC patients is stratified according to the initial stage at first presentation. Stage II patients in low risk situation (missing risk factors – see above) should not receive an adjuvant systemic therapy because of poor benefit (122). Patients with risk factors in stage II disease should be treated individualized, considering individual risk factors for recurrence, performance status, estimated benefit as well as good prognosis in curative operated situation and potential risks of chemotherapy (123). Klingbiel et al. recently showed, that for patients with stage II disease, a deficiency in MMR protein expression is of high prognostic relevance and a marker of a more favorable outcome and therefore these patients do not seem to profit from adjuvant chemotherapy (124). Therefore, the MSI status in stage II patients has to be taken into account, since patients with high frequency microsatellite instability do not benefit from fluorouracil-based chemotherapy. However, for patients at risk of recurrence and MSI-H status, an oxaliplatin containing regimen is a possible alternative (125, 126). Apart from that, recommended chemotherapy agents for stage II patients are capecitabine or 5-FU/leucovorin.

As already mentioned above, the therapeutic procedure in adjuvant setting of colon cancer is not clearly defined over all stages. In stage I, the international recommendation implies, that after curative surgery no further therapeutic procedures should be performed – survival rates are >90 percent and thus excellent. In stage II decision making is much more difficult and tailored to a patient's individual situation. Three large prospective trials have been performed investigating the role of adjuvant 5-FU based chemotherapy in stage II colon cancer. Two of them failed to show any survival benefit in stage II colon cancer patients (127, 128) and one described a marginal 3.6 percent overall survival benefit for adjuvant chemotherapy. In this study also node positive (means stage III) patients were included and after adjusting for stage

II the difference between both arms (chemotherapy vs. observation) also failed to be statistically significant (129). A combination therapy regime like FOLFOX – containing infusional Oxaliplatin and Leucovorin and 5-FU – also didn't show any survival benefits in several investigations (123, 130). Nonetheless, in a huge retrospective analysis of 115 100 stage II colon cancer patients, survival benefits were found to be associated with adjuvant chemotherapy, regardless of treatment regimen, patient age, or high-risk pathologic risk features (131). Given data support some clinicopathologic features to be associated with a lower survival – as there are T4 primary, poorly differentiated histology, signet ring and mucinous subtype, lymphovascular invasion, perineural invasion, bowel obstruction or perforation, less than 12 sampled lymph nodes, positive or nearly positive resection margins, high pre-surgical serum carcinoembryonic antigen (CEA) and occult nodal micrometastases (132-137). Nevertheless, given data about adjuvant chemotherapy in high risk stage II colon patients are also conflicting and no clear recommendation can be made because of missing prospective trials investigating the influence of adjuvant therapy in high risk stage II patients. Because of the obvious insufficiency of clinic-pathologic parameters several molecular factors have been investigated, but none of them was clearly capable of improving the predictive forecast whereas the prognostic value was more accurate. KRAS, BRAF, p53 mutation, as well as MSI are only an excerpt of investigated markers – however, the presence of MSI seems to be associated with a relative resistance to 5-FU and a lack of expression of the transcription factor CDX2 identified stage II patients who benefited from adjuvant chemotherapy (138, 139). All things considered no standard recommendation exists regarding the application of chemotherapy in stage II colon cancer patients. Therefore, a decision is hard to be made in each situation and has to be discussed with the patients, especially because of possible treatment toxicity, which has to be taken into account. Severe side effects (grade 3 or 4) are commonly seen in about 15 percent of patients treated with 5-FU chemotherapy alone and rise up to 20 percent in case of additional Oxaliplatin doses. What is notable is, that treatment-related death ranges from 0.5 to 1 percent, long term toxicity not included (140).

Stage III

In stage III patients the recommendations are more precise. After recovery from surgery adjuvant therapy should start within eight weeks, since given data show lower benefit in case of a too long delay after surgery (141, 142). The recommended therapy schedule in stage III CRC patients is a 6-month course of an oxaliplatin-based regimen and its superiority to fluoropyramidine-based regimen alone has been approved in several trials (121, 143-146). The average risk reduction of tumor recurrence and mortality in stage III CRC patients due to chemotherapy is about 20 to 30 percent. In case of contraindications to oxaliplatin, the fluoropyramidine containing therapy remains the standard, subject to microsatellite stability.

Stage III colon cancer means that tumor cells have spread to loco-regional lymph nodes. Surgical resection is principally the only potential curative option in colon cancer. However, beside the extension seen in the surgical specimen the major problem in further course of disease is the prevalence of so called micrometastases, which cannot be detected by traditional radiological investigations. Therefore, the current evidence recommends the application of adjuvant chemotherapy to reduce the risk of relapse. The most common used therapeutic regimens is FOLFOX. It contains Oxaliplatin 85mg/m² body surface, given on day 1, leucovorin 400mg/m² body surface day 1, 5-fluorouracil 400mg/m² body surface day 1 as a bolus injection and 5-flurouracil 2400mg/m² body surface day one administered over 46 hours as a continuous infusion per infusion pump system (147). The application of the chemotherapy should be repeated every two weeks for a period of 6 months or 12 cycles. There are several subtypes, like FOLFOX 4 or FOLFOX6 or mFOLFOX6, of the FOLFOX regimen, which differ in application dose or timetable – however they do not differ in efficacy. CAPOX or XELOX, containing Oxaliplatin 130mg/m² body surface administered on day 1 and Capecitabine 1250mg/m² body surface twice a day on day 1 to 14, is a good alternative in adjuvant treatment of stage III colon cancer patients and even seems to be slightly more effective as FOLFOX. The cycles are repeated every 3 weeks for 8 times. Capecitabine is a prodrug biotransformed into active 5-fluorouracil in the liver (148). There is some evidence, that Capecitabine, compared to infusional LV/5-FU, goes along with less severe neutropenia or even febrile neutropenia and less stomatitis. However, rates of polyneuropathy, hand foot syndrome and thrombocytopenia are more frequently seen in XELOX than in FOLFOX (146, 149). Recently, the duration of adjuvant chemotherapy was discussed at the ASCO 2017, as it could be shown, that the application of only 6 cycles of FOLFOX –3 months of therapy compared to usually used 6 months – is not associated with a

meaningful lower survival in low risk stage III tumors (T1 to T3 and N1; 3 year DFS 83,1% versus 83,3%) but goes along with a much lower rate of side effects, especially polyneuropathy. (data not published yet) However, other therapeutic agents, often used in palliative situation, like irinotecan, cetuximab or bevacicumab should not be used in adjuvant therapy because of evidenced lack of efficacy. Moreover, patients with MSI and contraindications for the use of Oxaliplatin are not treated with 5-fluorouracil alone, due to the same reasons as in stage II. There is substantial difference in individuals' course of tumor disease and currently it is not or hardly predictable which patients either in stage II or III will profit of an adjuvant chemotherapy.

Aim of the project

Recent evidence suggest that human cancers are stem cell diseases (150-154). Cancer cells with the properties of stem cells, known as cancer stem cells (CSCs), have the ability to self-renew, undergo multilineage differentiation, and survive adverse tissue microenvironment (155, 156). Moreover, studies in colon cancer have identified CSCs as capable of initiating tumor development (90, 91, 113). Several studies have suggested CD133 (PROM1), CD26 (DDP4) and CD44 as markers of tumor-initiating colon cancer cells (91, 150, 156). The aim of the current study was to investigate the prognostic as well as predictive role of several SNPs in the three mentioned genes in order to find more precise biomarkers for patients with stage II or stage III colon cancer. Thus, the individual respond to adjuvant treatment can probably be better predicted or at least prognostic groups, which could guide to adjuvant therapy management can be defined better.

Materials and methods

Potential SNPs

The genes CD44, CD133 and CD26 were selected as they had been shown to play a role in colon cancer development and/or progression. Polymorphisms known to play a role in tumor disease but not investigated in CRC were searched and chosen on public literature resources and databases including; NCBI-Pubmed, dbSNP, Ensembl, Pharmacogenomic-Knowledge-Base and GeneCards. Stringent and pre-defined selection criteria were used:

1) minor allel frequency ≥10% in Caucasians
2) polymorphisms that could influence a gene's function in a relevant way, either confirmed by
    a. published data or
    b. predicted function using Single-Nucleotide-Polymorphism database (F-SNP – offline) – similar database:
        https://www.ncbi.nlm.nih.gov/projects/SNP/
3) published clinical associations

Eligible patients

Between 1995 and 2011, 801 patients with histopathologically confirmed stage II (n=373) and III (n=428) colon cancer were consecutively recruited at the Division of Clinical Oncology, Department of Medicine, Medical University of Graz. The clinical stage according to UICC was assessed based on the resection specimen and the radiomorphologic presentation at the time of surgery. From 599 patients paraffin-embedded normal tissue adjacent to tumor samples was available for germline genetic testing. A total of 391 patients was treated with adjuvant 5-FU-based chemotherapy and 208 patients were treated with surgery alone. All patients were included in a colon cancer surveillance program, suggesting history and physical examination and CEA determination every 3 months for 3 years, every 6 months at years 4 and 5 and yearly at years 6–10 after surgery. Colonoscopy was performed at year 1 and thereafter every 3–5 years and x-ray of the chest and abdominal ultrasound or CT scans of chest and abdomen every 3–6 months for the first 5 years and x-ray of the chest and abdominal ultrasound yearly from year 6 to10. Patient data were collected retrospectively through chart review. This study has been approved by the Institutional Review Board (IRB) of the Medical University of Graz. All participants were Caucasians (1).

Isolation of genomic DNA and determination of SNPs

Tissue samples were stored at the Biobank of the Medical University of Graz (certified according to EN/ISO 9001:2008) Genomic DNA was extracted from paraffin-embedded normal tissue distant from the tumor to obtain germline DNA. Generally, the samples from the resection margins were used, and all tissue samples were re-evaluated by a board certified pathologist to ensure tumor-free tissue. DNA isolation was performed using the QIAamp DNA mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genotypes for CD44 (rs187116 A>G, rs7116432 A>G, rs353639 A>C, rs13347 C>T and rs187115 A>G) DPP4 (rs2268889 A>G, rs3788979 A>G, rs7608798 A>G) and CD133 (rs2240688 A>C), were centrally determined by 5′-exonuclease assay (TaqMan). Primer and probe sets were designed and manufactured using Applied Biosystems 'Assay-by-Design' custom service (Applera, Vienna, Austria). General TaqMan reaction conditions were documented according to the manufacturer of the assays. End-point fluorescence was measured in a Lambda Fluoro 320 plus plate reader (MWG Biotech AG, Ebersberg, Germany) using excitation/emission filters of 485/530 and 530/ 572 nm, respectively. The data were exported into Excel format and depicted and analyzed as scatter plots. In the plots, genotype groups were identified as separate and distinguishable clusters. As a control for consistency of the genotyping method, determination of genotypes was repeated in at least 96 samples. The rules of good laboratory and clinical practice were observed. The investigator analyzing the germline polymorphisms was blinded to the clinical data set (1).

Statistical analysis

The primary end point of the study was TTR, which was defined as the time from date of diagnosis of colon cancer to the date of first tumor recurrence. If a patient had not recurred, TTR was censored at the time of death or at the last follow-up. The secondary end point was overall survival (OS), which was defined as the time from date of diagnosis of colon cancer to the date of death from any cause. Allelic distribution of polymorphisms was tested for deviation from Hardy–Weinberg equilibrium using χ^2-test. The true mode of inheritance of the polymorphism tested has not been established yet and we assumed a dominant or recessive genetic model where appropriate. However, we couldn't find any differences between the dominant and the recessive model; therefore, we subsequently only used the dominant model. The association of polymorphisms with TTR and OS was analyzed using Kaplan–Meier curves and compared by log-rank test. Demographic and clinico-pathological features were included in multivariable analysis when a p value of <0.2 had been achieved in univariable analysis. In a stepwise backward multivariable Cox-regression analysis for TTR the features age, tumor location, tumor size, number of resected lymph nodes, lymphovascular-, vascular- and perineural invasion and stage were included. For OS the features age, tumor side, invasion depth, number of resected lymph nodes, tumor grade, lymphovascular-, vascular- and perineural invasion, and stage as well as application of adjuvant chemotherapy were included. HR and 95% confidence intervals were reported. Case-wise deletion for missing polymorphisms was used in univariable and multivariable analyses. A p-value <0.05 was considered to be statistically significant. All analyses were performed using SPSS for Windows (Version 22, SPSS Inc., Chicago, IL, USA). Statistical analyses were supported and partly performed by the Institute for Medical Informatics, Statistics and Documentation of the Medical University of Graz (1).

Results

Median age at time of diagnosis was 65 years. (range 27 to 95) There were more colon cancers in men than women and tumors occurred more frequently in the left side of the colon. The great majority of tumors had already been ingrown through the muscularis propria (T3). In general there were 237 lymph node negative (means stage II) and 362 lymph node positive (means stage III) patients. Among the lymph node positive cases, nearly two third of the patients had less than four positive lymph nodes (=N1). In 86 percent of all operated patients there were more than twelve lymph nodes available for pathologic investigation. Lymphangiosis (27,4 percent), haemangiosis (10 percent) as well as perineural invasion (2.5 percent) was detected only in a minority of investigated cases (1).

Tumor recurrence was observed in 185 (30.9%) patients with a probability of 5-year recurrence of 34.9% (standard deviation (sd) 2.2). Tumor recurred in 46 (19.6%) out of 235 stage II colon cancer patients and in 139 (38.2%) out of 364 stage III colon cancer patients. Adjuvant chemotherapy was administered in 82 (34.9%) in stage II versus 309 (84.9%) cases in stage III. The reasons for missing chemotherapy in stage III were diverse – among these, low clinical performance status and patients' refusal were the most common reasons for the missing treatment. Table 1 shows the baseline characteristics of the 599 patients included in the analysis and their association with TTR and OS.

The genotyping quality control provided a genotype concordance of 99%. Genotyping was successful in at least 71.6% of cases for each polymorphism analysed. (range 71.6 to 89.3%) In failed cases, genotyping was not successful because of limited quantity and/or quality of extracted genomic DNA. Baseline single nucleotide polymorphisms and their distribution are shown in table 2.

The allelic frequencies for 8 of 9 polymorphisms were within the probability limits of Hardy–Weinberg equilibrium (estimating, that allele and genotype frequencies remain constant from generation to generation), which is shown in table 3. For DPP4, rs2268889 the allelic frequency was not in the probability limit of Hardy-Weinberg equilibrium and the distribution of SNPs and occurence of either death or relapse is shown in table 4.

The SNP rs187116 in the gene CD44 showed no association with TTR in the univariate analysis (p=0.56). Neither showed the other tested gene variants, CD44 rs7116432 (p=0.93), DPP4 rs2268889 (p=0,34), CD44 rs353639 (p=0.65), DPP4 rs3788979 (p=0.70), DPP4 rs7608798 (p=0.11), CD44 rs13347 (p=0.09) and CD133 rs2240688 (p=0.66) a statistically significant association with TTR in the univariable analyses (Figure 1) (1).

Nine polymorphisms were investigated, among which the gene variant CD44 rs187115 showed a statistically significant association with TTR in univariable analysis. Patients carrying at least one G allele had a significantly reduced risk of recurrence compared to patients with the homozygous A/A variant (HR 0.70, 95% CI 0.50-0.95, p=0.036) in the dominant model. Patients carrying the homozygous A/A variant in SNP rs187115 had a probability of 5-year recurrence of 40.0% (sd 4.1) compared to patients carrying at least one G allele having a probability of 29.3% (sd 2.9).

The association with TTR remained significant in multivariable analysis after adjusting for age, tumor location, tumor invasion, count of operated lymph nodes, lymphangiosis, hemangiosis, neural invasion and stage (HR 0.67, 95% CI 0.48-0.94, p=0.019) (Table 5, figure 2) (1).

Death was observed in 168 (28.0%) out of 599 patients with a probability of 5-year survival of 71.7% (sd 2.1). The tested gene variants CD44 rs187116 (p=0.94), CD44 rs7116432 (p=0.52), DPP4 rs2268889 (p=0.99), CD44 rs353639 (p=0.75), DPP4 rs3788979 (p=0.59), DPP4 rs7608798 (p=0,20), CD44 rs187115 (p=0.73) and CD133 rs2240688 (p=0.94) did not show a statistically significant association with OS in the univariable analyses (Figure 3).

The SNP CD44 rs13347 showed a statistically significant association with OS in univariable analysis. Patients carrying at least one T allele in rs13347 had a significantly reduced risk of death compared to patients with the homozygous C/C variant (HR 0.62, 95% CI 0.42-0.93, p=0.019). Patients carrying the homozygous C/C in rs13347 had a probability of 5-year survival of 67.6% (sd 3.4) compared to patients carrying one C allele or the homozygous T/T 76.7% (sd 3.8). This result remained significant in multivariable analysis including age, tumor location, tumor invasion depth, count of operated lymph nodes, lymphangiosis, hemangiosis,

neural invasion, stage and application of adjuvant chemotherapy (HR 0.61, 95% CI 0.41-0.92, p=0.019) (Table 5, figure 4) (1).

Possible associations between clinicopathological parameters and the 2 prognostically relevant SNPs (rs13347 and rs187115) were further investigated. However, neither rs187115 nor rs13347 was associated with stage of disease or any other clinico-pathological parameter (Table 6). Because all other investigated SNPs showed no associations with TTR and OS, an association study for clinico-pathological parameters was not performed for these SNPs because of missing clinical relevance.

In order to evaluate the predictive role of the investigated SNPs an analysis for rs187115 and rs13347 and their association with chemotherapeutic outcome on TTR for rs187115 and OS for 13347 was done. In case of rs187115 the SNP was not a predictive marker for efficacy of chemotherapy (p=0.87). However, rs13347 showed a statistically significant association with chemotherapy, and thus it could be a predictive marker for chemotherapy (p=0.049) (figure 5) (1).

Discussion

Colon cancer is a common tumor entity and one of the leading causes of cancer related death worldwide. Like in other tumors one of the major prognostic factors is the tumor stage at diagnosis with a decrease of survival at a higher tumor stage. However, the inter-individual differences in prognostic outcome not only stage dependent. In recent years the knowledge about molecular mechanisms, including different signal transduction pathways, in the CRC's development has greatly increased. Looking at SNPs it becomes clear how individually profiled each human being can be. The molecular markers show their potential as predictive markers in the RAS mutation status for the prediction of efficacy of anti- EGFR-antibodies.

Molecular markers can be predictive, prognostic or even both, whereas prognostic markers identify patients with different risks of a specific outcome of the disease, either without a systemic therapy and/or associated with a differential outcome regardless of treatment. A prognostic marker normally does not determine the decision for a specific therapy and a predictive marker can predict differential efficacy of a particular therapy according to the marker status and therefore helps to define the therapeutic choice (157, 158).

Recently, the hypothesis that a specific subset of CSCs is responsible for tumor metastasis has been investigated in CSCs isolated from human colon cancer specimens. Pang et al. identified a subpopulation of CSCs from human tumors that is capable of forming metastasis in an orthotopic animal model. The unique and most important finding of this study was the capacity of CD26+ cells to form liver metastasis when injected into the cecum of mice irrespective of CD133 or CD44 expression. However, coexpression of CD133 and CD44 did increase the metastatic capacity of CD26+ cells. In contrast, CD26- cells were not capable of forming liver metastasis, irrespective of whether CD133 or CD44 was positive or negative. Furthermore, dissociated tumor cells have been exposed to 5-FU or oxaliplatin in vitro and analyzed for CSC subsets. Treatment was associated with either drug decreased cell viability or enhanced apoptosis and caused significant tumor shrinkage. At eight weeks posttreatment, 80% or more of viable cancer cells in the tumors treated with chemotherapy were CD133+CD26+, and the tumors started to regrow after eight weeks. These data suggested that chemotherapeutic treatments that fail to eliminate CSCs may enrich the metastatic CSC subpopulations (74). There is substantial germline genetic variability within the genes for CD26 (DPP4), CD133 (PROM1) and CD44 (CD44), including multiple single nucleotide polymorphisms (SNPs). Recent data suggest that SNPs may be significant, previously unrecognized factors in cancer progression/metastasis and chemoresistance (159, 160). DNA sequence variations in CSC genes could influence protein activity or even production, which

could cause the differences of metastatic capacity and chemoresistance. Recently, Zhou et al. analyzed 260 breast cancer patients and 232 healthy controls and found an association between the CD44 Ex2+14 A>G gene variant in the CD44 intron 1 and CD44 expression and breast cancer development (161). Furthermore, Vazquez et al. described an association between the CD44 rs187115 SNP and response to chemotherapy and overall survival in patients with soft-tissue sarcoma (162). To date, there is only limited data investigating PROM1 and DPP4 SNPs for cancer risk/outcome and chemoresistance. It is possible but unlikely that one SNP would have a dramatic effect on clinical colon cancer outcome and chemoresistance. Therefore, a multigenic approach, which assesses the combined effects of DPP4, PROM1 and CD44 gene variations may detect synergistic interactions between individual SNPs and enhance the predictive power for colon cancer metastasis and chemoresistance.

The CD44 gene is located on the short arm of chromosome 11, is 50 kilobases (kb) long and consists of 20 exons, 12 of which are involved in splicing mechanisms (163). It is a transmembrane glycoprotein that fulfills several functions in cell biology as adhesion, signalling and division by binding several ligands, including hyaluronic acid (HA). Cell to cell communication as well as signal transduction is influenced by CD44. Moreover, it interacts with the EGFR (epidermal growth factor receptor) (164) and shows activity in the regulation of the inflammatory response (165, 166). A strong CD44 expression in neoplastic crypts and advanced adenomas is indicative of its role in tumorigenesis of the gastrointestinal tract. Additionally, it has been identified as a CSC marker in colon cancer (74, 113). However, CD44 is not exclusively found in colon cancer, since its isoforms are heterogeneously expressed in breast cancer and correlate with breast cancer subtypes (165). Nonetheless, the exact effect of altered CD44 expression remains unclear and has to be clarified more clearly, particularly because given data suggest an important role in human cancers.

Winder et al. investigated the role of CD44 rs187116 in gastric cancer patients. The group found that patients, harboring at least one G allele (A/G or G/G genotype) had a median TTR of 2.1 years, compared to 7.0 years in patients homozygous for A allele (p=0.022).

Although the precise functional and biological significance of these polymorphisms remained unclear, Winder et al. assumed that CD44 intronic polymorphisms could have an impact on the regulation of gene splicing events (5). Similar findings were illustrated by the group of Suenaga, who also found the presence of CD44 rs187116 A/G or G/G genotypes significantly associated with worse disease-free survival than in those with the A/A genotype (P = 0.039)

(167). In our investigation we could neither show a statistically significant correlation with neither TTR nor OS in CRC patients. This might be traced back to different cancer type on the one hand or investigated populations on the other hand. But as there is no other investigation in colon cancer despite ours up to now it is likely that this SNP plays no role in colon cancer. Similar results were found in CD44 rs7116432 where Winder et al. could show a significant association with TTR in gastric cancer but we failed to show a correlation in colon cancer.

CD44 rs353639 was associated with the risk of bladder cancer development in an Indian population, investigated by Verma et al. They found a marginal risk in the dominant model, GT + TT of rs353639 ($p = 0.044$) and reduced risk in variant allele T ($p = 0.040$), especially increased in smokers carrying variant genotype, TT of CD44rs353639 G/T ($p = 0.038$). Their study showed that rs353639 goes along with a marginal risk for bladder cancer susceptibility, whereas rs4755392 and rs13347 had reduced risk of bladder cancer and rs187115 and rs187116 had no effect on bladder cancer susceptibility in their population (168). As already shown in rs7116432 and rs187116, we also found no association with neither TTR nor OS in colon cancer. However, for rs13347 we could show an association with survial. Tulsyan et al. investigated rs353639 in breast cancer patients and found a correlation with clinical tumor size. Using F-SNP, they described an altered transcriptional regulation for rs353639 polymorphism and concluded that CD44 rs353639 genetic variants may have significant effect in breast cancer prognosis (1, 169).

Apparently, the same SNPs play a different role in either different populations or different cancer entities. Nevertheless, this is speculative because there is not enough data to prove this consideration. Nonetheless, a review of Zhang et al. came to the conclusion, that SNPs in CD44 might not represent risk factors for cancer but the group had to admit, that their findings required further validation in better designed studies with larger sample sizes (170). The SNPs on DPP4 rs2268889, rs7608798 and rs3788979 could be shown to affect the PSA level in men, which is a positive predictive factor for prostatic cancer (171). Therefore we tested these SNPs in colon cancer as predictive or prognostic marker respectively; however all three failed to show any association with TTR or OS. CD133 rs2240688 has been investigated in lung and gastric cancer. The expression of CD133 is significantly correlated with either the development as well as prognosis of non small cell lung cancer (172). The group of Liu et al. found poorer prognosis associated with *rs2240688* A>C variant compared to rs2240688AA genotype. Higher HRs for associations between CD133 rs2240688 polymorphism and OS were observed in patients with adjuvant chemotherapy and radiotherapy for curative intent

compared with patients without adjuvant chemotherapy or without radiotherapy. Moreover Liu et al. found rs2240688 AA genotype compared to the variant AC/CC genotypes associated with a statistically increased risk of lung cancer in a recessive model (173, 174). Interestingly, Jia et al. found the A/C or C/C genotypes of CD133 rs2240688 associated with a significantly decreased risk of gastric cancer compared to the A/A genotype (p=0.023) (175). It could be observed in a study by Cheng et al. that the rs2240688 A-to-C transition leads to a new binding location of the microRNA has-miR-135a/b, which might be of high importance in modulating the effect of the SNP on CD133 expression (176). It could be shown, that SNPs in the 3'-UTR influence the control of mRNA stability and efficiency by regulating miRNA, including miR-34a, −101,–128, −137 and −138 (174, 177). Therefore we considered, that rs2240688 in CD133 could influence the function of CD133 and thereby consequentially prognosis in colorectal cancer too. However, we could not find any association with survival parameters in CRC patients (1).

Among the nine SNPs investigated in the present study, rs13347 and rs187115 showed a prognostic value in stage II and III colorectal cancer patients. Recently, the role of SNP rs13347 as well as rs187115 was investigated in non-small cell lung cancer (NSCLC) patients. Interestingly, the group of Liu et al. could not find any association between the SNP rs13347 and NSCLC risk, whereas rs187115 was significantly associated with survival. Allele G carriers had a significantly higher rate of bone metastasis ($p<0.001$) and a more advanced tumor stage ($p=0.001$) compared to carriers of the allele A. The survival rates for patients with AA genotype were significantly higher than for patients with the AG+GG genotypes ($P<0.001$) (178). This is contrary to our findings in CRC patients, as we were unable to show an association between rs187115 and altered survival rates. On the other hand, we could demonstrate an association for rs187115 regarding TTR, which was significantly higher in patients with the AG or GG genotype compared to patients with the homozygous AA genotype (1).

Stracquadanio et al. investigated the role of CD44 SNPrs187115 in pancreatic adenocarcinoma patients and could demonstrate an up to 2.38-fold increased risk for tumor-related death for mutated genotypes AG/GG in their cohort, which is also contrary to our findings in colorectal cancer (179). However, both Stracquadanio and Liu included metastasized patients in their cohorts, as opposed to our study. Wu et al. showed that the C to T base change of rs13347 disrupts the binding site for hsa-mir-509-3p and increases the transcriptional activity of the *CD44* gene. They showed that patients with rs13347 CT and TT genotypes harbored significantly higher *CD44* mRNA levels compared to carriers of the

rs13347CC genotypes. In their study, the variant genotypes CT and TT increased an individual's susceptibility to CRC by the 1.6 fold, compared with rs13347 CC. Interestingly, they also discovered a more profound risk effect of this polymorphism in tumor stages III and IV (180). In our study, however, the homozygous genotype rs13347 CC was associated with a reduced survival rate compared to patients harboring the CT or TT genotype (1). An explanation for this diversity may be related to the fact that we did not include stage IV patients in our cohort. Therefore, the SNP rs13347 may actually be of different prognostic value in adjuvant versus metastatic situations analogous to the role of microsatellite instability status in distinct colorectal cancer stages (29).

The stability of genomic DNA allows easy handling and processing. In addition, SNP analysis is relatively robust between platforms and laboratories, which helps to reduce inter-laboratory variation. Knowledge about the clinical effects of CSC SNPs in colon cancer is essential because it leads to a better understanding of colon cancer behavior. Moreover, the existence of gene variants, prone to develop chemoresistance provides a strategy for stratifying patients for risk of metastatic colon cancer and chemoresistance, which could allow a more individual treatment plan. Another benefit might be a decreased incidence of tumor relapse by placing patients on adjuvant therapy that would not be initiated in current circumstances. Conversely, it may ultimately be possible to avoid treating patients at low risk, thus eliminating the morbidity associated with adjuvant therapies (181).

Limitations

There are several limitations of our study that have to be taken into account. On the one hand it has a retrospective design; thus, a selection bias cannot be fully excluded. On the other hand, frequencies of polymorphisms may vary between different ethnicities. Moreover, albeit the number of investigated cancer patients was quite large the number of SNPs in the cohort was not only naturally smaller but also because of limited quality of blood probes. Another point that has to be considered is the predictive value of SNP rs13347 in case of chemotherapy. Albeit this result might be clinically important it has to be admitted, that this result may be influenced by stage, respectively that the factor stage has a great influence on survival and chemotherapy application, as stage II patients have a better prognosis per se and receive much less frequently an adjuvant chemotherapy.

Conclusion

In conclusion, we discovered rs187115 as being an independent prognostic biomarker regarding TTR as well as rs13347 concerning OS, in stage II and III colorectal cancer patients. Nevertheless, prospective trials are needed to validate these promising genetic biomarkers in colon cancer patients (1).

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Tables

Table 1: Baseline patient characteristics and their association with TTR and OS in univariable analysis(1)

Parameter	N	%	TTR		OS	
			HR (95% CI)	p-value	HR (95% CI)	p-value
Gender						
Male	330	55.1	1 (reference)	0.861	1 (reference)	0.300
Female	269	44.9	0.98 (0.73-1.30)		0.85 (0.63-1.16)	
Age						
in years			1.02 (1.00-1.03)	0.012	1.05 (1.04-1.07)	<0.001
Tumor location						
Left	375	62.6	1 (reference)	0.075	1 (reference)	0.058
Right	224	37.4	1.31 (0.97-1.75)		1.35 (0.99-1.83)	
Lymph node operated						
≤12	84	14.0	1 (reference)	0.868	1 (reference)	0.734
> 12	515	86.0	1.04 (0.69-1.56)		0.93 (0.63-1.39)	
Tumor size						
T1 & T2	35	5.8	1 (reference)	<0.001	1 (reference)	<0.001
T3	434	72.5	2.62 (0.97-7.10)		1.80 (0.74-4.42)	
T4	130	21.7	5.28 (1.81-14.59)		4.22 (1.69-10.56)	
Lymph node involvement						
N0	237	39.6	1 (reference)	<0.001	1 (reference)	<0.001
N1	229	38.2	1.60 (1.09-2.34)		1.41 (0.97-2.06)	
N2	133	22.2	4.17 (2.89-6.03)		2.94 (2.02-4.29)	
Tumor grade						
G1 & G2	418	69.8	1 (reference)	0.279	1 (reference)	0.017
G3	181	30.2	1.19 (0.87-1.61)		1.46 (1.07-1.99)	
Lymphovascular invasion						
No	435	72.6	1 (reference)	0.007	1 (reference)	0.136

Yes	164	27.4	1.51 (1.12-2.05)		1.28 (0.93-1.78)	
Vascular invasion						
No	539	90.0	1 (reference)	<0.001	1 (reference)	<0.001
Yes	60	10.0	2.41 (1.67-3.49)		2.06 (1.38-3.08)	
Perineural invasion						
No	584	97.5	1 (reference)	<0.001	1 (reference)	0.060
Yes	15	2.5	3.84 (2.09-7.27)		2.07 (0.97-4.41)	
Clinical stage						
II	235	39.2	1 (reference)	<0.001	1 (reference)	<0.001
III	364	60.8	2.40 (1.72-3.35)		1.92 (1.38-2.67)	
Adjuvant chemotherapy						
No	208	34.7	1 (reference)	0.236	1 (reference)	0.110
Yes	391	65.3	1.21 (0.88-1.65)		0.78 (0.57-1.06)	

Table 1: N=number of cases, TTR=time to recurrence, OS=overall survival, HR=hazard ratio, CI=confidence interval; reproduced from Stotz et al. with permission of Anticancer Research

Table 2: Baseline Single nucleotide polymorphisms (SNP) and distribution

Name	Description	Coding	Count (N %valid)	Valid	Missing
SNP1	rs187116	0=CC (wildtype) 1=CT (heterozygous) 2=TT (mutated)	149 (27.9%) 257 (48.1%) 128 (24.0%)	534	65
SNP2	rs7116432	0=AA (wildtype) 1=AG (heterozygous) 2=GG (mutated)	198 (40.2%) 216 (43.8%) 79 (16%)	493	106
SNP3	rs2268889	0=GG (wildtype) 1=GA (heterozygous) 2=AA (mutated)	207 (44.1%) 190 (40.5%) 72 (15.4%)	469	130
SNP4	rs353639	0=AA (wildtype) 1=AC (heterozygous) 2=CC (mutated)	312 (58.3%) 188 (35.1%) 35 (6.5%	535	64
SNP5	rs3788979	0=GG(wildtype) 1=GA (heterozygous) 2=AA (mutated)	384 (74.7%) 116 (22.6%) 14 (2.7%)	514	85
SNP6	rs13347	0=CC (wildtype) 1=CT (heterozygous) 2=TT (mutated)	251 (58.5%) 154 (35.9%) 24 (5.6%)	429	170
SNP7	rs187115	0=AA (wildtype) 1=AG (heterozygous) 2=GG (mutated)	183 (37.7%) 237 (48.9%) 65 (13.4%)	485	114
SNP8	rs7608798	0=GG (wildtype) 1=GA (heterozygous) 2=AA (mutated)	212 (44.6%) 203 (42.7%) 60 (12.6%)	475	124

SNP9	rs2240688	0=AA(wildtype) 1=AC (heterozygous) 2=CC (mutated)	268 (54.1%) 181 (36.6%) 46 (9.3%)	495	104

Table 2: SNP=single nucleotid polymorphism, A=adenine, G=guanine, C=cytosine, T=thymine; N=number of cases

Table 3: SNPs and Hardy-Weinberg Equilibrium (HWE) and frequency of events

			HWE		
			Total	X^2	p-value (df=1)l
rs187116	CC	Count	99		
	CT	Count	180		
	TT	Count	89		
	Total	Count	368	0.163	0.687
rs7116432	AA	Count	138		
	AG	Count	146		
	GG	Count	53		
	Total	Count	337	1.879	0.170
rs2268889	GG	Count	149		
	GA	Count	123		
	AA	Count	51		
	Total	Count	323	8.390	0.004
rs353639	AA	Count	218		
	AC	Count	132		
	CC	Count	26		
	Total	Count	376	0.948	0.330
rs3788979	GG	Count	267		
	GA	Count	81		
	AA	Count	12		
	Total	Count	360	3.378	0.066
rs13347	CC	Count	167		
	CT	Count	113		
	TT	Count	21		
	Total	Count	301	0.099	0.753
rs187115	AA	Count	122		
	AG	Count	166		
	GG	Count	55		
	Total	Count	343	0.014	0.907
rs7608798	GG	Count	147		
	GA	Count	150		

	AA	Count	47		
	Total	Count	344	0.773	0.379
rs2240688	AA	Count	186		
	AC	Count	134		
	CC	Count	31		
	Total	Count	351	0.931	0.335

Table 3: SNP=single nucleotid polymorphism, HWE= Hardy-Weinberg Equilibrium, A=adenine, G=guanine, C=cytosine, T=thymine;

Table 4: Event rate (relapse or death) and SNPs

			Relapse			Death		
			No	Yes	Total	No	Yes	Total
Event rate	Total	Count	414	185	599	431	168	599
rs187116	CC	Count	99	50	149	108	41	149
	CT	Count	180	77	257	184	73	257
	TT	Count	89	39	128	92	36	128
	Total	Count	368	166	534	384	150	534
rs7116432	AA	Count	138	60	198	142	56	198
	AG	Count	146	70	216	160	56	216
	GG	Count	53	26	79	53	26	79
	Total	Count	337	156	493	355	138	493
rs2268889	GG	Count	149	58	207	149	58	207
	GA	Count	123	67	190	133	57	190
	AA	Count	51	21	72	53	19	72
	Total	Count	323	146	469	335	134	469
rs353639	AA	Count	218	94	312	230	82	312
	AC	Count	132	56	188	134	54	188
	CC	Count	26	9	35	24	11	35
	Total	Count	376	159	535	388	147	535
rs3788979	GG	Count	267	117	384	275	109	384
	GA	Count	81	35	116	86	30	116
	AA	Count	12	2	14	12	2	14
	Total	Count	360	154	514	373	141	514
rs13347	CC	Count	167	84	251	170	81	251
	CT	Count	113	41	154	121	33	154
	TT	Count	21	3	24	23	1	24
	Total	Count	301	128	429	314	115	429
rs187115	AA	Count	122	61	183	140	43	183
	AG	Count	166	71	237	162	75	237
	GG	Count	55	10	65	50	15	65
	Total	Count	343	142	485	352	133	485

rs7608798	GG	Count	147	65	212	153	59	212
	GA	Count	150	53	203	150	53	203
	AA	Count	47	13	60	49	11	60
	Total	Count	344	131	475	352	123	475
rs2240688	AA	Count	186	82	268	194	74	268
	AC	Count	134	47	181	132	49	181
	CC	Count	31	15	46	34	12	46
	Total	Count	351	144	495	360	135	495

Table 4: A=adenine, G=guanine, C=cytosine, T=thymine;

Table 5: SNP rs13347 and SNP rs187115 and their association with TTR and OS in multivariable analysis (dominant model)(1)

Parameter	**SNP rs13347**				**SNP rs187115**			
	TTR		**OS**		**TTR**		**OS**	
	HR(95%CI)	**p-value**	**HR(95%CI)**	**p-value**	**HR(95%CI)**	**p-value**	**HR(95%CI)**	**p-value**
SNP rs13347					n.d.	n.d.	n.d.	n.d.
C/C	1 (reference)	0.076	0.61 (0.41-0.92)	0.019				
C/T or T/T	0.72 (0.50-1.04)							
SNP rs187115	n.d.	n.d.	n.d.	n.d.				
A/A					1 (reference)	0.019	1 (reference)	0.921
A/G or G/G					0.67 (0.48-0.94)		0.98 (0.68-1.42)	
Age	1.02 (1.01-1.04)	0.012	1.05 (1.03-1.07)	<0.001	1.02 (1.01-1.04)	0.009	1.05 (1.03-1.07)	<0.001
tumor location	n.d.	n.d.	n.d.	n.d.			n.d.	n.d.
left side					1 (reference)	0.048		
right side					0.71 (0.50-0.997)			
Tumor invasion								
T1&T2	1 (reference)		1 (reference)		1 (reference)		1 (reference)	
T3	2.61 (0.95-7.16)	0.063	2.79 (0.99-7.91)	0.054	2.94 (1.08-8.02)	0.036	2.73 (0.996-7.50)	0.051
T4	5.83 (2.06-16.48)	0.001	7.51 (2.58-21.84)	<0.001	6.41 (2.27-18.06)	<0.001	8.02 (2.83-22.77)	<0.001

Haemangiosis							n.d.	n.d.
No	1 (reference)		1 (reference)		1 (reference)			
yes	1.85 (1.17-2.92)	0.008	1.61 (0.97-2.67)	0.065	1.84 (1.20-2.83)	0.005		
Stage								
Stage II	1 (reference)		1 (reference)		1 (reference)		1 (reference)	
Stage III	2.05 (1.39-3.04)	<0.001	2.55 (1.62-4.01)	<0.001	2.088 (1.439-3.029)	<0.001	2.86 (1.87-4.37)	<0.001
Adjuvant Chemotherapy	n.d.	n.d.			n.d.	n.d.		
No			1 (reference)				1 (reference)	
yes			0.51 (0.33-0.80)	0.003			0.51 (0.34-0.78)	0.002

Table 5: SNP=single nucleotid polymorphism, N=number of cases, TTR=time to recurrence, OS=overall survival, HR=hazard ratio, CI=confidence interval, A=adenine, G=guanine, C=cytosine, T=thymine, n.d.=not done; reproduced from Stotz et al. with permission of Anticancer Research

Table 6: Baseline patient characteristics and their association with SNP rs13347 and SNP rs187115 in univariable analysis(1)

Parameter	SNP 13347					SNP 187115				
	CC	CT/TT	Miss	OR (95% CI)	p-value	AA	AG/GG	Miss	OR (95% CI)	p-value
Gender										
Male	131	107	92	1 (reference)	0.104	108	161	61	1 (reference)	0.211
Female	120	71	78	0.72 (0.49-1.07)		75	141	53	1.26 (0.87-1.83)	
Age										
in years				1.00 (0.98-1.02)	0.887				1.00 (0.98-1.01)	0.772
Tumor location										
right	89	71	64	1 (reference)	0.350	68	111	45	1 (reference)	0.929
left	162	107	106	0.83 (0.56-1.23)		115	191	69	1.02 (0.70-1.49)	
Lymph node operated										
≤12	35	20	29	1 (reference)	0.409	22	43	19	1 (reference)	0.488
> 12	216	158	141	1.28 (0.71-2.30)		161	259	95	0.82 (0.48-1.43)	
Tumor invasion										
T1 & T2	18	10	7	1 (reference)	0.697	13	20	2	1 (reference)	0.846
T3	179	133	122	1.34 (0.60-2.99)		132	225	77	1.11 (0.53-2.30)	
T4	54	35	41	1.17 (0.48-2.82)		38	57	35	0.98 (0.43-2.19)	
Lymph node										

involvement										
N0	100	77	60	1 (reference)	0.627	74	123	40	1 (reference)	0.437
N1	94	67	68	0.93 (0.60-1.43)		63	117	49	1.12 (0.73-1.70)	
N2	57	34	42	0.78 (0.46-1.30)		46	62	25	0.81 (0.50-1.31)	
Tumor grade										
G1 & G2	181	121	116	1 (reference)	0.356	127	212	79	1 (reference)	0.852
G3	70	57	54	1.22 (0.80-1.85)		56	90	35	0.96 (0.65-1.44)	
Lymphovascular invasion										
No	181	130	124	1 (reference)	0.833	134	219	82	1 (reference)	0.865
Yes	70	48	46	0.96 (0.62-1.47)		49	83	32	1.04 (0.69-1.57)	
Vascular invasion										
No	224	160	155	1 (reference)	0.830	162	272	105	1 (reference)	0.592
Yes	27	18	15	0.93 (0.50-1.75)		21	30	9	0.85 (0.47-1.54)	
Perineural invasion										
No	243	174	167	1 (reference)	0.563	176	295	113	1 (reference)	0.341
Yes	8	4	3	0.70 (0.21-2.36)		7	7	1	0.60 (0.21-1.73)	
Stage										
II	100	75	60	1 (reference)	0.634	73	123	39	1 (reference)	0.966
III	151	103	110	0.91 (0.62-1.34)		110	179	75	0.97 (0.66-1.40)	
Adjuvant										

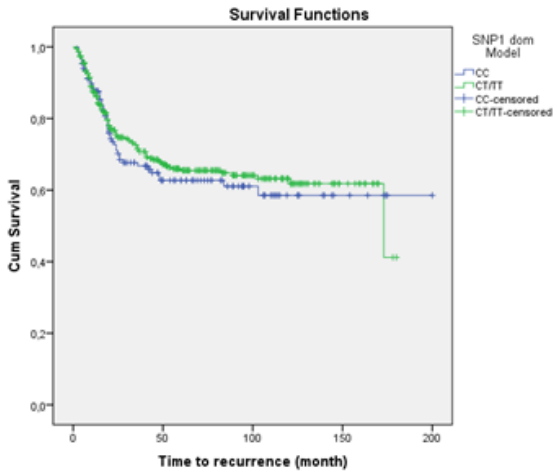
chemotherapy										
No	91	59	58	1 (reference)	0.506	66	108	34	1 (reference)	0.946
Yes	160	119	112	1.15 (0.77-1.72)		117	194	80	1.01 (0.69-1.49)	

Table 6: SNP=single nucleotid polymorphism, OR=odds ratio, CI=confidence interval, Miss=missing, A=adenine, G=guanine, C=cytosine, T=thymine; reproduced from Stotz et al. with permission of Anticancer Research

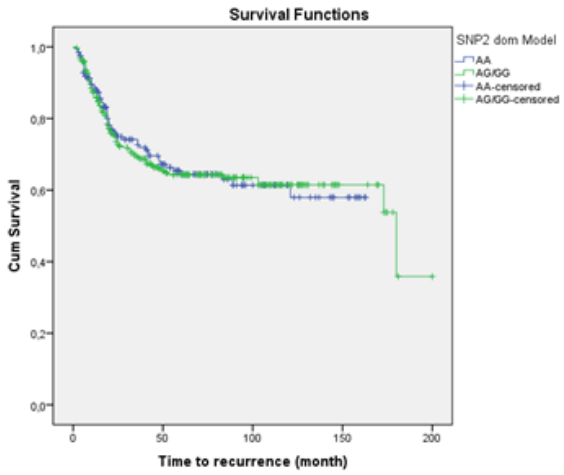
Figures

Figure 1: Dominant model of SNP 1 to 6 and 8 and 9 and association with TTR

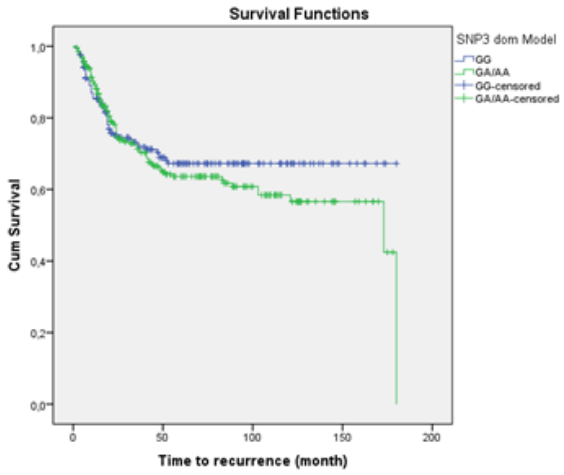
rs187116 dominant model (SNP1)



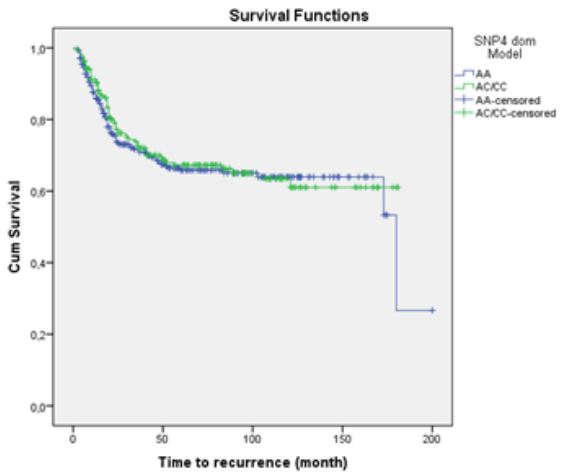
rs7116432 dominant model (SNP2)



rs2268889 dominant model (SNP3)



rs353639 dominant model (SNP4)



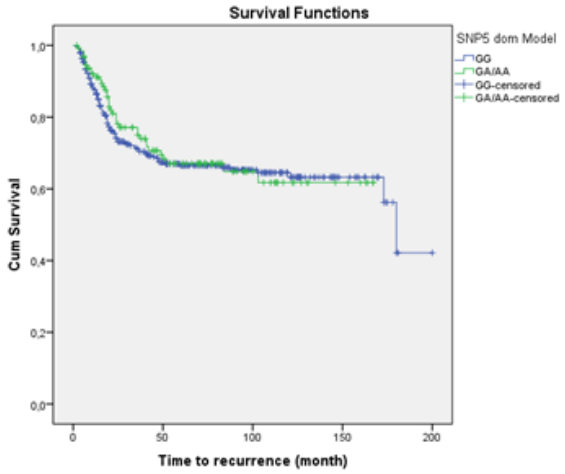
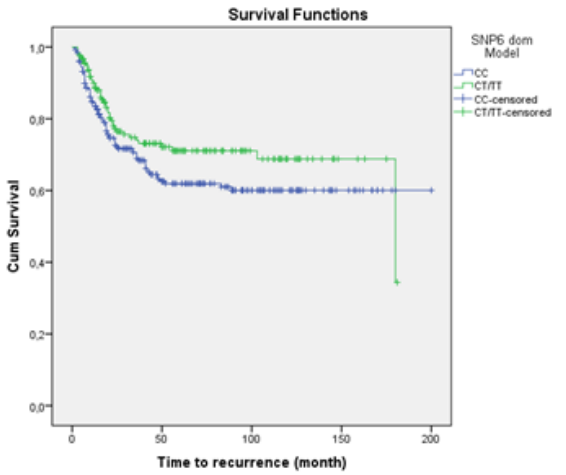
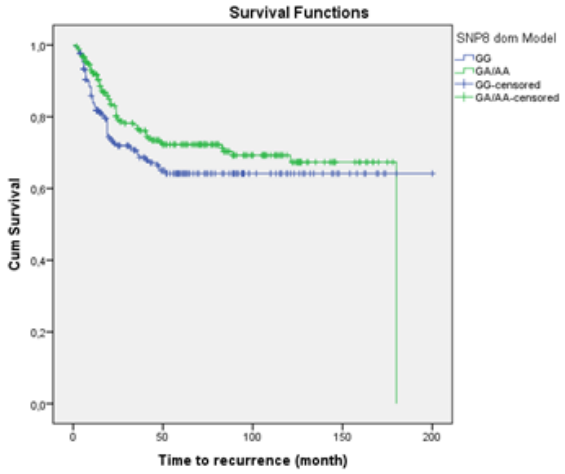
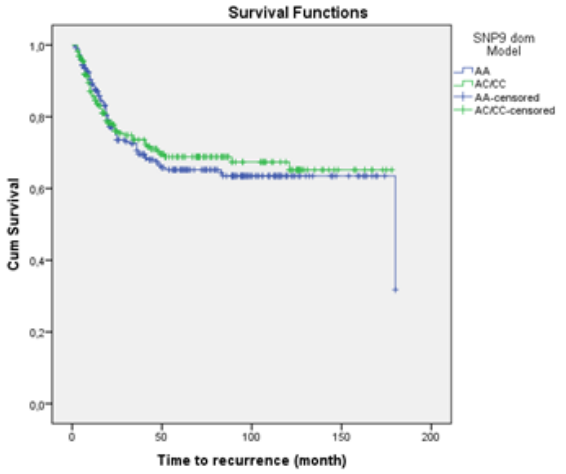
rs3788979 dominant model (SNP5)	rs13347 dominant model (SNP6)
	
rs7608798 dominant model (SNP8)	rs2240688 dominant model (SNP9)
	

Figure 1: SNP=single nucleotide polymorphism, TTR=time to recurrence, dom=dominant, A=adenine, G=guanine, C=cytosine, T=thymine,

Figure 2: Association of SNP rs187115 and TTR (n=599)(1)

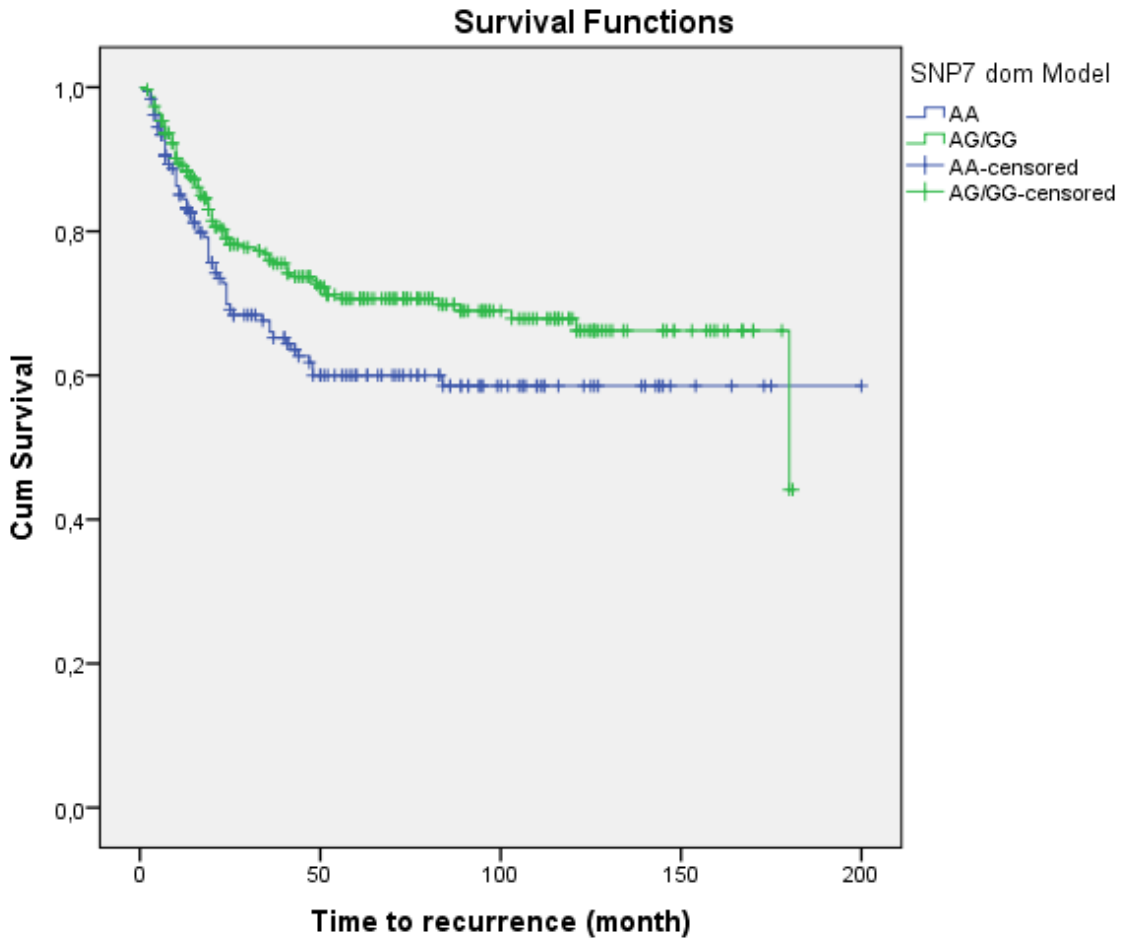
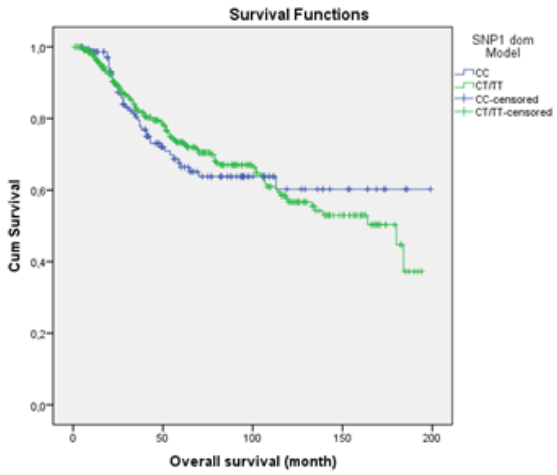


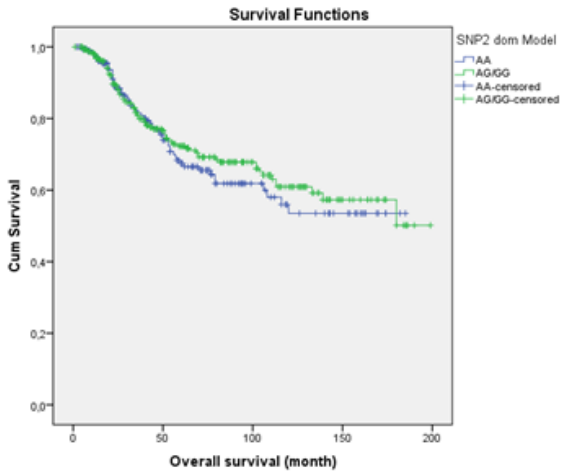
Figure 2: SNP=single nucleotide polymorphism, TTR=time to recurrence A=adenine, G=guanine, C=cytosine, T=thymine, dom=dominant; reproduced from Stotz et al. with permission of Anticancer Research

Figure 3: Dominant model of SNP 1 to 5 and 7 to 9 and their association with OS

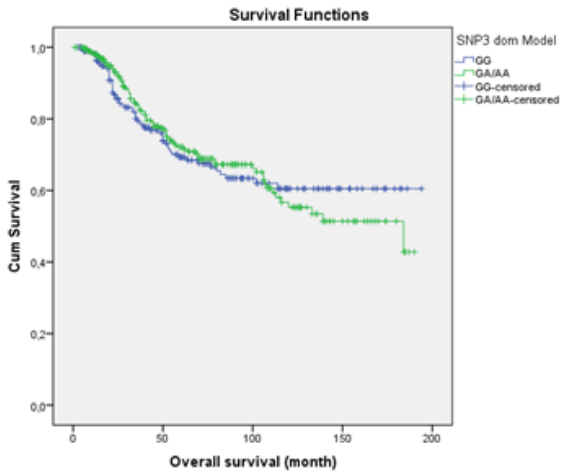
rs187116 dominant model (SNP1)



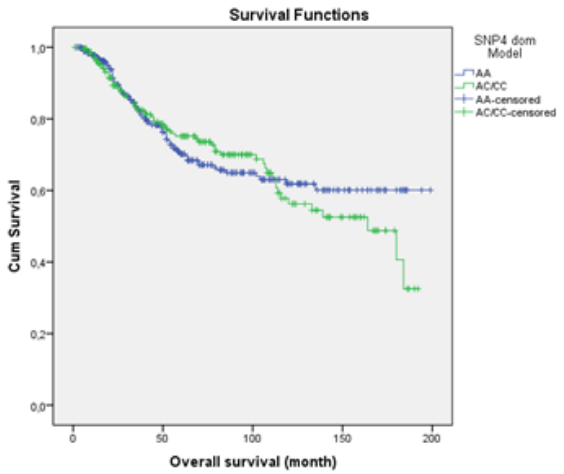
rs7116432 dominant model (SNP2)



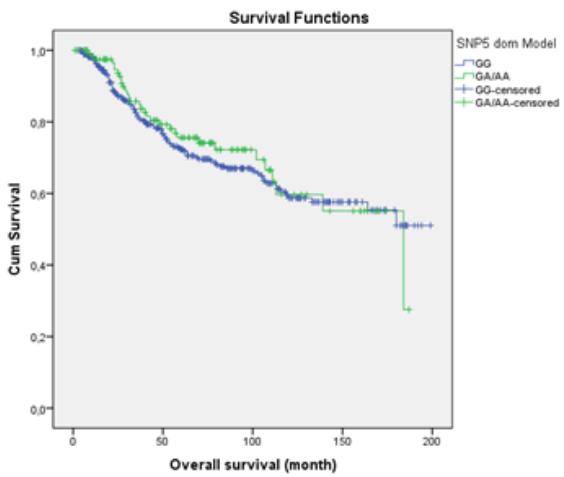
rs2268889 dominant model (SNP3)



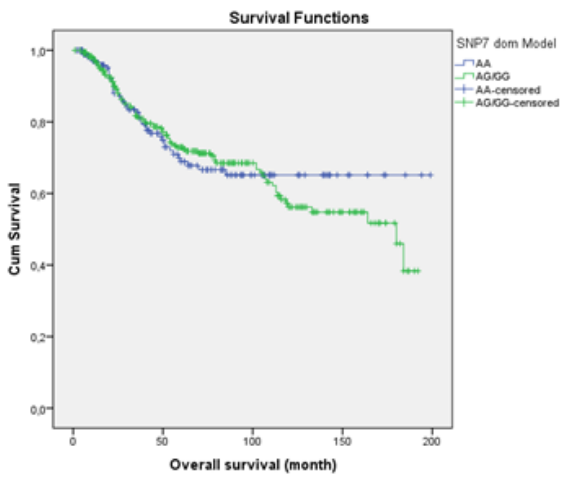
rs353639 dominant model (SNP4)



rs3788979 dominant model (SNP5)



rs187115 dominant model (SNP7)



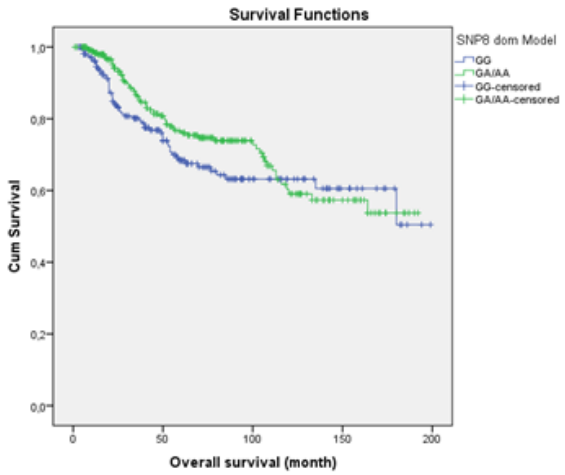
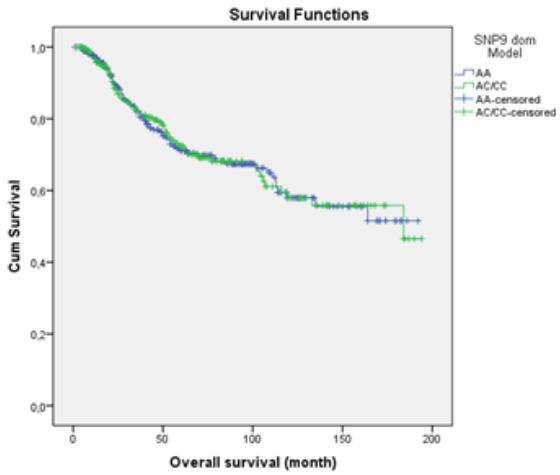
rs7608798 dominant model (SNP8)	rs2240688 dominant model (SNP9)
	

Figure 3: SNP=single nucleotide polymorphism, OS=overall survival, dom=dominant, A=adenine, G=guanine, C=cytosine, T=thymine,

Figure 4: Association of SNP rs13347 with OS (n=599)(1)

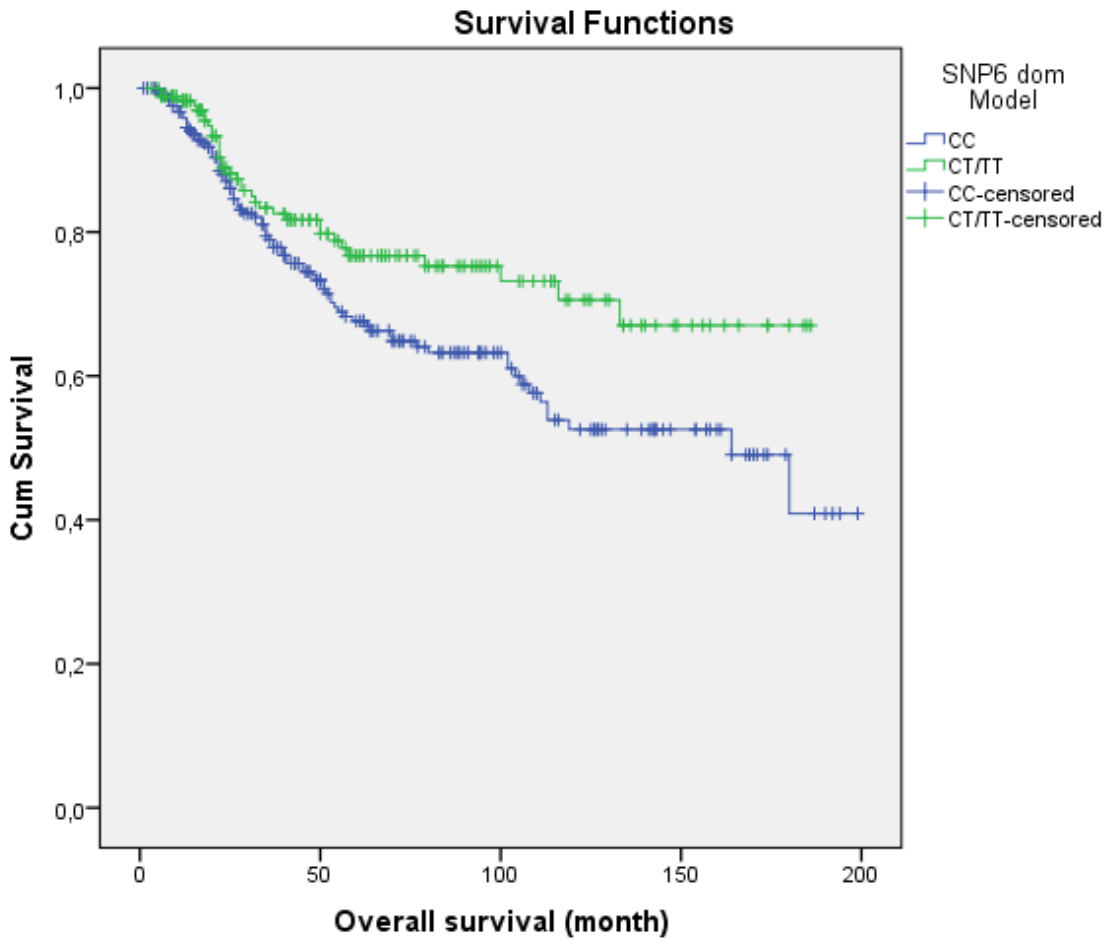


Figure 4: SNP=single nucleotide polymorphism, OS=overall survival, A=adenine, G=guanine, C=cytosine, T=thymine, dom=dominant; reproduced from Stotz et al. with permission of Anticancer Research

Figure 5: Association of SNP rs13347 with Chemotherapy and OS (n=599)

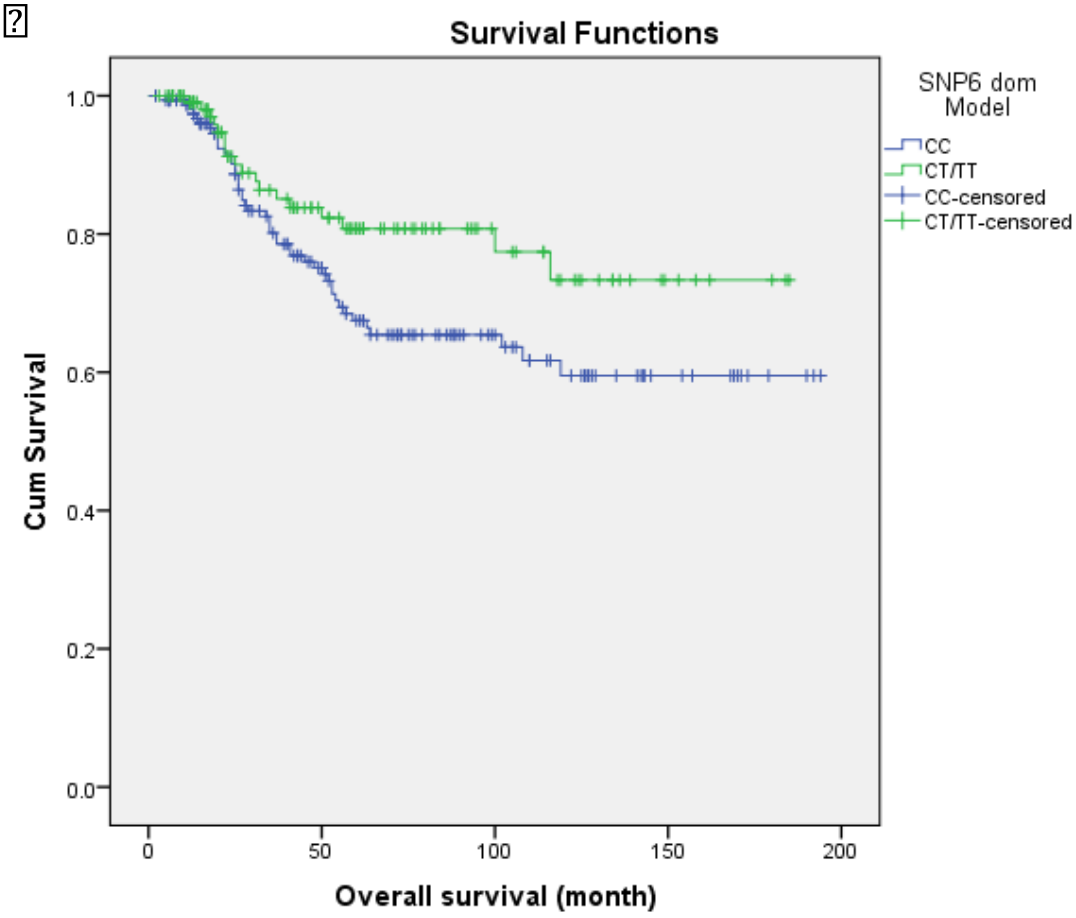


Figure 5: SNP=single nucleotide polymorphism, OS=overall survival, dom=dominant, A=adenine, G=guanine, C=cytosine, T=thymine, dom=dominant;