SUMMARY AND DISCUSSION

Colorectal cancer (CRC) is a major healthcare problem in the Western world and is associated with high mortality rates.[www.cijfersoverkanker.nl] CRC is a complex and heterogeneous disease, characterized by the development of precursor lesions (adenomas) of which a small percentage will eventually progress into a cancer (adenoma-carcinoma sequence). Early work of Morson7 followed by Vogelstein8-10 provided a progression model for CRC in which key events during colorectal carcinogenesis were described. Mutations in APC, KRAS and TP53 but also losses of chromosomes 17 and 18q are important features of this model. Later on, two different forms of genetic instability were added to this progression model, chromosomal instability (CIN) (~85% of all CRCs) and microsatellite instability (MSI) (~15% of all CRCs), each having different underlying causes and clinical outcome.11,12 More recently, it also has become clear that epigenetic alterations are involved in CRC development, resulting in the recognition of the so-called CpG island methylator phenotype (CIMP).13-15

Whereas the adenoma-carcinoma sequence has provided us with a first concept of colorectal carcinogenesis, it has also created a paradigm in which the terms polyps and adenomas have long been used as synonyms. Although in 1985 Muto already described a type of lesion in the colorectum that was termed “small flat adenoma”, the notion that nonpolypoid sporadic CRC exist, has long been ignored and interpreted as reflecting a non-Western entity. This has changed only quite recently due to publication of large series of nonpolypoid lesions in the US and post-colonoscopy CRCs (interval CRC) can occur after colonoscopy.16,17

Colonoscopy has a central role in every CRC prevention program, as it allows detection and removal of precursor lesions thereby successfully preventing CRC.18 However, it does have limitations19-21 and post-colonoscopy CRCs (interval CRC) can occur after colonoscopy.12,16 Nonpolypoid lesions are suspected to be an important cause of these interval cancers19,21 for two main reasons. First, nonpolypoid lesions may more easily go undetected during colonoscopy (in particular when the endoscopist is less experienced and/or bowel preparation is suboptimal). Second, this different phenotype may be associated with a different underlying biology. At least a sub-group of nonpolypoid lesions have been associated with a more aggressive behavior and are considered more likely to contain advanced histology.5,17,18

As tumor phenotypes are, for a substantial part, driven by their genotypes, the aim of this thesis was to investigate the molecular characteristics of nonpolypoid adenomas. To this end we collected a large well-defined sample collection in which all adenomas were classified according to the Paris classification22 and analyzed these with multiple molecular techniques.

Knowledge on molecular characteristics of nonpolypoid lesions

At the start of this project little was known about the molecular characteristics of nonpolypoid adenomas, conflicting results were reported on some molecular changes such as KRAS and BRAF mutations. In Chapter 2, we provided a systematic review of all studies published up to 2011 that investigated molecular changes in nonpolypoid lesions. We specifically focused on studies that investigated the KRAS, BRAF or APC mutation status, MSI, CIMP status, or methylation of single genes known to be important in CRC development. Meta-analyses were performed for all studies investigating KRAS, APC or BRAF mutation status and MSI status showing a trend towards less KRAS and APC mutations and more BRAF mutations in nonpolypoid lesions compared to polypoid ones.

More specifically, we found indications that some subtypes of nonpolypoid lesions, that is depressed (0-IIc) and lateral spreading type non-granular (LST-NG) neoplasms, as well as early (T1) nonpolypoid carcinomas, are consistently associated with less KRAS mutations than their polypoid counterparts, which might argue for a distinct molecular pathway in these subgroups. We observed no difference in MSI frequencies between polypoid and nonpolypoid lesions. Only two studies investigated CIMP, precluding any conclusions regarding this feature. In general, this review showed that among the different studies large heterogeneity existed. The exact cause of this heterogeneity was unclear, but it may be related to methodological issues including small sample sizes, heterogeneous definition of nonpolypoid adenomas and a possible selection bias. Therefore the obtained results from our meta-analyses should be interpreted with caution.

This meta-analysis represented a first attempt to clarify the molecular biology of nonpolypoid lesions and identified the existing gaps in the current knowledge on molecular changes in nonpolypoid lesions. Our major finding was the eminent need for molecular studies containing large numbers of well-characterized nonpolypoid lesions. Therefore we collected a large well-characterized series of nonpolypoid adenomas and compared these with polypoid adenomas for several molecular characteristics (e.g. mutation, copy number alterations, hypermethylation).

Different mutation frequencies in nonpolypoid adenomas

In Chapter 3 we investigated the mutation status of 14 genes (BRAF, NRAS, KRAS, PIK3CA, PIK3R1, EGRF, PSEN, MAP2K4, SMAD4, FBXW7, CTNNB1, STK11, PDGFRB and APC), known to be involved in CRC development, in a large multi-centre series of well-defined nonpolypoid and polypoid adenomas. Mutations in KRAS, BRAF, NRAS, FBXW7 and CTNNB1 showed similar frequencies in both phenotypes, whereas mutations in APC were more frequent in polypoid adenomas compared to nonpolypoid adenomas. For all other genes no mutations were observed in any of the adenomas investigated.

The genes studied in this chapter are involved in a number of different signal transduction pathways including the RAS-RAF-MAPK pathway, PI3K-AKT pathway and the Wnt-signaling pathway. The lower APC mutation rate in nonpolypoid adenomas compared to polypoid adenomas suggests
that disruption of the Wnt-pathway may be less important or may occur via different mechanisms in nonpolypoid adenomas. Furthermore, in contrast to previous observations our results in this large well-defined sample set indicate that there is no significant association between the different morphological phenotypes and mutations in key genes of the RAS-RAF-MAPK pathway.

Common chromosomal alterations in nonpolypoid adenomas
Chromosomal alterations are known to play a role in CRC carcinogenesis, however, the number of studies investigating this phenomenon in nonpolypoid lesions is lagging behind. In Chapter 4 we investigated DNA copy number alterations in polypoid and nonpolypoid adenomas using an arrayCGH platform with unprecedented high resolution. In addition, we also investigated occurrence of the alternative genetic instability pathway, MSI in nonpolypoid adenomas and polypoid adenomas.

Our results showed that patterns of DNA copy number changes differed between the two phenotypes, with significantly more frequent loss of 5q14.3 and 5q15-q31.1 in nonpolypoid adenomas, whereas losses of 1p36.32-p35.3, 10q25.3, 17p12, and chromosome 18 were more frequent in polypoid adenomas (false discovery rate<0.2). The occurrence of MSI in both nonpolypoid and polypoid adenomas was similar and very low (~1%).

Loss of chromosome 5q has been associated with an aggressive clinical course in CRC\textsuperscript{20-22} consistent with the supposedly more aggressive behavior of nonpolypoid adenomas. On the other hand, it could also indicate that a specific group of the colorectal carcinomas is originating from nonpolypoid adenomas instead of polypoid adenomas.

As the 5q15-q31.1 region harbors the APC locus, we combined our previous results on APC mutations with the 5q/APC gene copy number data. This combined analysis showed that nonpolypoid adenomas have significantly more frequent chromosome 5q (locus of APC) loss and simultaneously less frequent APC mutations compared to polypoid adenomas. Interestingly, loss of 5q and lack of APC mutation, as reported here for nonpolypoid adenomas, were described before in ulcerative colitis-associated CRC\textsuperscript{23,24} Moreover, next to APC a cluster of interleukin genes associated with inflammation was located at this lost 5q region. An initial exploration of the possible association between 5q loss and inflammation indicated that tumor-infiltrating lymphocytes were more abundant in the stroma of nonpolypoid adenomas compared to that of polypoid adenomas. Based on our findings, we suggest that similar pathways might be involved in the biology of nonpolypoid colorectal neoplasia and inflammatory bowel disease (IBD) associated CRC. These results warrant further investigation into the possible involvement of inflammation in the underlying molecular biology of nonpolypoid lesions.

The role of methylation and CIMP in nonpolypoid adenomas
In Chapter 5 we investigated the promoter hypermethylation status of 11 genes known to be relevant in CRC pathogenesis (MLH1, O6MGMT, APC, p14\textsuperscript{ARF}, p16\textsuperscript{INK4A}, RASSF1A, RASSF2A, GATA-4, GATA-5, CHFR and HLF) and CIMP. Two large independent cohorts, both containing over 200 adenomas (~100 nonpolypoid adenomas and ~100 polypoid adenomas) were used, in which the methylation status of all genes was related to phenotype, location, and dysplasia.

We found no consistent differences in promoter hypermethylation for the investigated genes between nonpolypoid and polypoid adenomas in both cohorts. Also the CIMP status was not different between both phenotypes. Given the results obtained, methylation markers remain promising as candidate screening markers for both nonpolypoid and polypoid adenomas.

Interestingly, we did find a significant difference in methylation between distinct locations and sizes for RASSF1A and CHFR, respectively. Methylation of RASSF1A was more frequently in the distal colon in both cohorts, suggesting that methylation of RASSF1A represents an (early) event that differs between distal and proximal tumorigenesis. CHFR methylation was significantly more frequent in large adenomas (>10mm) compared to small ones (<10mm) in both cohorts. Therefore, methylation of CHFR might be associated with malignant progression of adenomas.

Methylation of the Wnt-signaling pathway genes in colorectal adenomas
As described above we observed less APC mutations in nonpolypoid adenomas and concurrently more chromosomal loss of the APC gene locus (Chapter 4). To further complement our understanding of Wnt-pathway disruption, we also investigated the APC promoter hypermethylation status (Chapter 5). However, in a substantial part of all adenomas no APC disrupting event was observed (mutation, loss or methylation). This suggests that alternative mechanisms, such as methylation mediated silencing of upstream Wnt-pathway antagonists, may be involved in activation of the Wnt-signalling pathway. In Chapter 6, we therefore investigated DNA promoter methylation patterns of four Wnt-pathway antagonists, SFRP2, WIF-1, DKK3 and SOX17, in colorectal cancer cell lines, colorectal carcinomas and both non-polypoid and polypoid adenomas.

Increased methylation of all genes was found in the majority of colorectal cancer cell lines and carcinomas compared to normal controls. Moreover also in both types of adenomas high methylation levels of upstream Wnt-antagonists were observed indicating that this alternative mechanism for Wnt-signalling pathway activation is already employed in adenomas. When comparing polypoid to nonpolypoid adenomas, however, lower levels of methylation of WIF1 and DKK3 were found in the latter group. In addition, a positive relation between APC mutation and methylation of both WIF-1 and DKK3 methylation was observed in adenomas (p<0.05). This further substantiates the potential difference in Wnt-pathway disruption between polypoid and nonpolypoid adenomas.

Clinical effects of adjuvant active specific immunotherapy (ASI) in patients with and without MSI
Adjuvant therapy in stage II and III CRC with active specific immunotherapy has been explored by Vermorken and colleagues\textsuperscript{25} This therapy implies administering an autologous tumor cell vaccine,
derived from their own sterilized tumor cells, to CRC patients after resection of the primary tumor. ASI was shown improve recurrence-free survival of patients with CRC. At the time the ASI trial was conducted, however, awareness on the biologic heterogeneity of CRC and its possible clinical implications was still limited. Histologically, a clear difference in immune response between MSI and microsatellite stable (MSS) colon tumors is seen. MSI tumors often display poor or mucinous differentiation and contain more lymphocytic infiltrate,²⁶,²⁷ potentially resulting from an immune reaction against neoantigens formed by frameshift mutations in protein-coding sequences in MSI tumors. If true, microsatellite status could well affect the outcome of therapies that actually strive to modulate the immune response. Therefore in Chapter 7 we retrospectively investigated the association between response to ASI treatment and MSI status in CRC. Patients with MSI tumors had significantly fewer recurrences and prolonged disease free survival than those with MSS tumors, irrespective of treatment arm and tumor stage. Patients with MSS Dukes C tumors who received ASI treatment showed a significantly improved recurrence-free survival compared with controls. ASI treatment did not improve recurrence-free interval or disease free survival for patients with MSS Dukes C tumors. We conclude that the clinical benefit, measured as recurrence-free survival, from adjuvant ASI treatment of patients with CRC was restricted to patients with MSS Dukes B tumors.

Future perspectives
In this thesis we studied the molecular changes in nonpolypoid adenomas and compared these with those observed in “conventional” polypoid adenomas. When trying to link a specific phenotype to a genotype, correct phenotypic classification is very important. For this work we used a large, well-classified sample collection of nonpolypoid and polypoid adenomas, all classified according to the Paris classification.¹⁹

We have shown that molecular differences exist between nonpolypoid and polypoid adenomas (Figure 1), indicating nonpolypoid adenomas should be regarded as a separate identity in CRC development. Current models for CRC carcinogenesis (e.g. the model described by Vogelstein) are mainly based on the conventional polypoid adenomas (polyps) and are describing the most frequent molecular changes of only these lesions. As we and others found that nonpolypoid adenomas have different molecular changes, current models for colorectal carcinogenesis are not covering the complete spectrum of CRC development and are therefore in need of adjustment. Without adjustments, the role of novel or alternative cancer pathways may be ignored or underestimated when investigating CRC development. Our results also showed that already known CRC carcinogenesis pathways could be affected by distinct mechanisms, arguing for further generalization of current models.

Figure 1: Schematic overview of the molecular findings in this thesis. The grey blocks represent the different molecular events that were investigated in this thesis. Events observed more frequently in nonpolypoid lesions are located more to the left in the figure, whereas events more common in polypoid lesions are located more to the right side. Events that are not different between the two phenotypes are located at the middle.

Methylation: no consistent differences in promoter hypermethylation for the investigated genes or CIMP between nonpolypoid and polypoid adenomas could be observed, except, in a small cohort, for WIF-1 and DKK3, which both showed lower methylation levels in nonpolypoid adenomas compared to polypoid adenomas.

Mutations: APC mutations were significantly less frequently found in nonpolypoid adenomas compared to polypoid adenomas. For mutations in KRAS no difference could be observed in our own cohort, however, in literature there are indications that some nonpolypoid subtypes (depressed lesions (0-IIc), LST-NG as well as early (T1) nonpolypoid carcinomas) harbor less KRAS mutations compared to polypoid ones. In our cohort only few BRAF mutations were found and no significant difference was observed as was also the case for NRAS, FBXW7 and CTNNB1.

MSI: The occurrence of MSI in both nonpolypoid and polypoid adenomas was similar and very low (~1%).

DNA copy number alterations: loss of chromosome 5q was significantly more frequent in nonpolypoid adenomas compared to polypoid adenomas, whereas losses of 1p, 10q, 17p and chromosome 18 were more frequent in polypoid adenomas. Common alterations in CRC, including chromosome 20q and 13 gain, were not different between nonpolypoid and polypoid adenomas.
The work presented here clearly proved that molecular differences exist between polyloid and nonpolyloid adenomas, but only provided limited insights into the underlying mechanisms. However, one of the molecular changes, specifically observed in nonpolyloid adenomas, could be linked to inflammation. A potential implication of this observation would be that patients with IBD or other inflammatory diseases are in need of better screening for nonpolyloid lesions. Moreover this possible link may provide a basis for new therapies or interventions targeting the inflammatory process thereby reducing the development of nonpolyloid lesions. Further investigations are needed to substantiate this potential link and explore the potential clinical implications.

The findings described in this thesis may also have implications for CRC screening programs, which will commence this year (2013) in The Netherlands. The current algorithm for this screening program consists of a faecal immunochemical test (FIT) that, when positive, is followed by a colonoscopy, and is expected to result in a reduction in the mortality rates of CRC.15-19 Moreover, it is expected that detection rates of adenomas and early carcinomas will increase and rates of tumors with metastasis will decline.

Based on the observations that nonpolyloid adenomas may exhibit more aggressive behavior and may significantly contribute to interval CRC it is important that all endoscopists participating in the screening are aware of the existence of nonpolyloid lesions. Increasing awareness and training was shown to be an effective way for increasing the detection rate of nonpolyloid adenomas.15 In this way more of the potentially more aggressive nonpolyloid lesions can be removed. To be able to conduct reliable research regarding the frequency, underlying molecular mechanisms and clinical implications of nonpolyloid neoplasia, a correct classification, robust enough to be applicable in daily routine, is of utmost importance. Along these lines, the findings described in this thesis can be substantiated and extended. Moreover, increased detection and better classification of nonpolyloid lesions will also yield new insights of specific subtypes of nonpolyloid lesions. In this thesis we observed potential differences between certain subtypes: however due to the small number no solid conclusions could be drawn. In conclusion, the implementation of the CRC screening program, together with the increased awareness of nonpolyloid lesions, will not only lead to a better understanding of nonpolyloid lesions in general but also to more insight in the different nonpolyloid subtypes. Ultimately, this knowledge will provide a solid basis for potential adaptations of the current screening algorithms regarding patients with (increased risk for) nonpolyloid neoplasia.

At present much effort goes into the identification and validation of molecular markers that could further improve current screening algorithms. In our opinion, it is very important that the value of these novel molecular screening markers for detection of nonpolyloid adenomas is also thoroughly investigated. This means that all potential markers need to be validated in a well-characterized series of nonpolyloid lesions comprised of sufficient number of all distinct subtypes as well. In this thesis we showed that in general DNA hypermethylation of tumor suppressor genes does not appear highly related to the (non)polyloid phenotype. Given these results, methylation markers remain promising candidate CRC screening markers for both polyloid as well as nonpolyloid adenomas.

Concluding, the results described in this thesis further substantiate the molecular differences between nonpolyloid and polyloid adenomas. In addition, a potential link with a distinct underlying biology was found, in which nonpolyloid adenomas appeared more associated with inflammation. These results underline that nonpolyloid neoplasia should be considered as a separate entity in the spectrum of colorectal cancer development. Increased awareness and more optimal classification of nonpolyloid adenomas is urgently needed to further investigate both the underlying molecular biology and the subsequent clinical consequences of nonpolyloid neoplasia.

REFERENCES


**CHAPTER 8 SUMMARY & DISCUSSION, NEDERLANDSE SAMENVATTING**

**Chapter 8**

**Moleculaire karakterisering van nonpolypoide colorectale adenomen**

Dikke darmkanker, ook wel bekend als colorectaal carcinoma, is een groot probleem in Westerse landen. Veel mensen die de ziekte krijgen overlijden aan de gevolgen hiervan. Dikke darmkanker is een complexe en heterogene ziekte waarvan de exacte oorzaken onbekend zijn. De ziekte begint vaak met een goedadoorwaaide gezwel (voorlopers laesie) in de darm, ook wel adénoom genoemd. Een klein deel van alle adénomen zal zich uiteindelijk ontwikkelen tot een kwaadaardige tumor, ook wel carcinoom genoemd. Dit proces noemt men ook wel de adénoom-carcinoom route en deze werd ongeveer 40 jaar geleden voor het eerst beschreven.

De ontwikkeling van nieuwe technieken in het begin van de jaren 90, zorgde ervoor dat er vele moleculaire veranderingen zijn gevonden die een rol spelen in deze adénoom-carcinoom route. Bijvoorbeeld mutaties in genen, waardoor de functie van een gen verandert. Een ander voorbeeld zijn chromosomale afwijkingen die vaak leiden tot een abnormale hoeveelheid (een toe- of afname) van genen die op deze chromosomen liggen. Een laatste voorbeeld is het uitzetten van genen door aan het genetisch materiaal (DNA) een extra molecuul toe te voegen, zogenoemde DNA methylering. Al deze veranderingen leiden er toe dat de cellen minder goed kunnen luisteren naar de signalen uit de omgeving en daardoor ongecontroleerd kunnen gaan delen, wat uiteindelijk tot kanker kan leiden.

In het klassieke model van de ontwikkeling van darmkanker, waarin een adénoom uitgroeiert tot een carcinoom, wordt er vanuit gegaan dat alle adénomen de vorm hebben van een poliep (een champignonachtige uitgroei in de darmholte). Dit is ook de reden dat de termen poliep en adénoom vaak door elkaar kunst worden gebruikt. Echter, al in 1985 maakten onderzoekers in Japan melding van adénomen die niet de vorm van een poliep hadden. Ze beschreven een vlak adénoom, dit noemt men ook wel een nonpolypoide colorectale adénoom of neoplasie (NP-CRN). Geruime tijd werd dit omschreven als een Japanse/Oosters fenomeen. Pas toen Japanse onderzoekers hun kennis over de opsporing van deze vlakke adénomen deelden met Westerse collega’s werden ook hier vlakke adénomen gevonden. Tegenwoordig is er geen twijfel meer over het bestaan van vlakke adénomen.

Een colonoscopie is een endoscopisch onderzoek van de dikke darm met behulp van een camera. Dit onderzoek speelt een belangrijke rol in ieder darmkanker preventie programma. Met dit onderzoek kunnen adénomen opgespoord en verwijderd worden, daarmee wordt voorkomen dat een adénoom uitgroeiert tot een kwaadaardige carcinoom. Helaas worden tijdens dit onderzoek soms toch adénomen gemist waardoor er een kleine kans bestaat dat een patiënt kort na dit darmonderzoek toch darmkanker krijgt. Dit noemt men een interval kanker. Vlakke adénomen kunnen een belangrijke oorzaak zijn van deze interval kankers. Ten eerste zijn deze vlakke adénomen minder goed zichtbaar bij een colonoscopie, omdat ze niet uitsteken in de darmholte zoals poliepen doen. Een goede training van de arts is daarom noodzakelijk om deze adénomen te detecteren. En