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The Role, Significance and Applicability of Aberrant Crypt Foci in Clinical Practice

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1. Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related mortalities in the Western world, with over 1.2 million new cases and over 0.6 million deaths being recorded in 2008 [1].

Major risk factors of CRC are personal history of precursor lesion, inflammatory bowel disease, age (about 90% of cases occur after age 50) and family history of CRC or a genetic susceptibility to the development of CRC resulting from DNA mutations. It is estimated that approximately 15% of CRC cases develop as a result of inherited factors and 5-10% of them result from known genetic syndromes, e.g., familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal carcinoma (HNPCC), while most cases of CRC occurs sporadically (70-80%) [2]. In patients with inherited genetic factors CRC occurs early in life, usually before 40 years of their age, while in sporadic cases cancer develops after 40 years of age with the highest incidence between 60 and 70 years of age. CRC remains asymptomatic for years. Symptoms develop insidiously and are frequently present for months, sometimes years, before being diagnosed. If colon tumors are not identified and removed at the precancerous or adenoma stage, the disease gradually progresses into carcinoma stage where cancer cells invade the wall of the intestine and distant organs [1].

There are different approaches and strategies concerning how to reduce the mortality due to CRC. The surgical and chemotherapeutic treatment of CRC is usually costly, painful and the prognosis is not promising. Therefore, in clinical practice efforts have been directed toward identification and removal of precancerous lesions. Screening programs, which are based upon detection and removal of visible polypoid adenomas, have been implemented in a number of countries on nationwide scale.

Global cancer statistics shows that CRC-related mortality has been decreasing in Western countries due to improved treatment and early detection, which indicates that screening

program is one of the important steps in reducing the mortality due to CRC [1]. However, although screening programs are promising and represent one of the important steps in reducing the mortality due to CRC, reports demonstrate that there is still 25% of false-negative results due to flat or depressed precancerous lesions, which are commonly missed during conventional colonoscopy [3].

Recently, another promising approach has been demonstrated. It is directed toward identification of aberrant crypt foci (ACF), intermediate biomarkers predictive for CRC. The association of ACF with CRC is supported by shared histological and molecular features of ACF with colonic polyps and adenomas [4-7].

The aim of the present chapter is to summarize experimental and clinical results regarding morphological, histological and molecular characteristics of ACF with emphasis on current progress in the knowledge of CRC development. The role, significance and applicability of ACF in clinical practice is also presented and discussed.

2. Aberrant crypt foci (ACF)

ACF are the first lesions in multistep development of CRC, which can be seen on the colon surface with aid of magnification and/or dye.

ACF were first identified in 1987 by Bird on whole unembedded colon of carcinogen treated mouse [8]. Colon was fixed, stained with methylene blue and observed under low-magnification (10-40x M) stereomicroscope [9; 10]. This simple and rapid methodological approach enabled visualization of all crypts on the surface of the colon mucosa. Since their first identification numerous studies investigating morphology, distribution, histology and molecular characteristics of ACF have been performed. In 1991 reports on identification of ACF in the human colon were published. ACF were identified under a dissecting microscope after methylene blue staining on the mucosal surface of both formalin-fixed human colon resections and fresh (unfixed) colon resections [11-13].

Based on morphological appearance of crypts on the colon surface crypts can be regarded as normal or aberrant. Aberrant crypts can be observed as single altered crypt or as a cluster of altered crypts that form a focus termed ACF [8; 14; 15].

It is important to keep in mind that ACF is a term that denotes topographic or endoscopic observation. ACF can be identified as clusters of altered crypts in unembedded colon mucosa (fixed or fresh) under magnification after visualization by different dyes. In studies using animal models ACF are usually observed under stereomicroscope on whole colon mucosa that is fixed flat (to prevent excessive unevenness while viewing) and stained with methylene blue [9; 10]. In clinical practice ACF can be observed *in vivo* endoscopically with aid of dye spray (methylene blue or indigo carmine) using high magnification colonoscopy [6; 7; 16].

ACF is not a histological diagnosis. Structural and cytological features of ACF can be recognized or confirmed only after histological examination. However, at the same time it is noteworthy to mention that lesions seen in histologic sections of colon without prior topographic identification on colon surface can not be termed ACF.

To better understand the histological background of ACF as well as molecular alterations recognized and described at this stage of colon carcinogenesis, the next section is brief overview of histological criteria and classification of ACF, histologically denoted as colorectal intraepithelial lesions [17; 18].

3. Histological characteristics of ACF

Histologically, ACF are heterogeneous group of intraepithelial lesions that exhibit variable features, ranging from almost normal or mild atypia to severe dysplasia. Based on their histological characteristics they can be divided into three main categories [7; 14]:

1. ACF that are almost histologically normal,
2. ACF with hyperplastic crypts and
3. ACF with dysplastic crypts.

According to World Health Organization ACF are histologically classified into two groups, i.e. ACF with hyperplastic crypts and ACF with dysplastic crypts [19].

It has been demonstrated that the majority of observed ACF, including ACF identified in patients with sporadic CRC, are classified as almost histologically normal (Figure 1). These ACF are composed of crypts with almost normal histological appearance. The major histologic difference that distinguishes this type of ACF from normal crypts is slightly enlarged crypt diameter. The crypt diameter in this type of ACF measures up to 1.5 times the diameter of a normal crypt. They show no other histological or molecular alterations and they even spontaneously regress. Accordingly, it was found that this type of ACF has no clinical diagnostic value [7; 14].

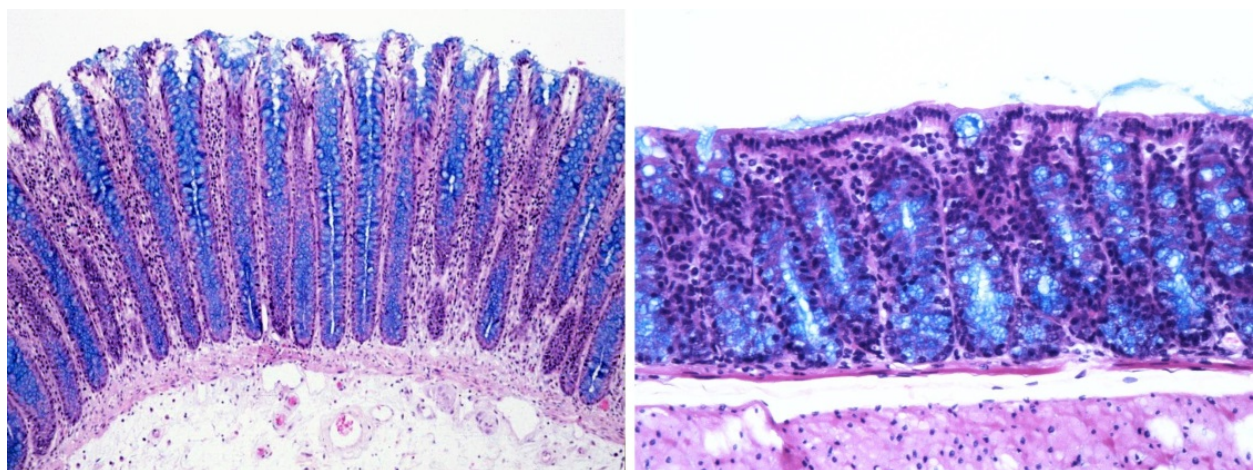


Figure 1. A normal human (left) and rat (right) colorectal mucosa. Crypts are parallel. The mucin is stained blue (Kreyberg trichrom stain).

On the other hand, other two groups of ACF have been found to have potential clinical value as biomarker predictive for CRC risk [7; 14].

4. ACF with hyperplastic crypts (hyperplastic intraepithelial lesions)

Hyperplastic epithelial lesions are composed of mixture of goblet and absorptive cells with enlarged or sometimes crowded nuclei without stratification (Figure 2).

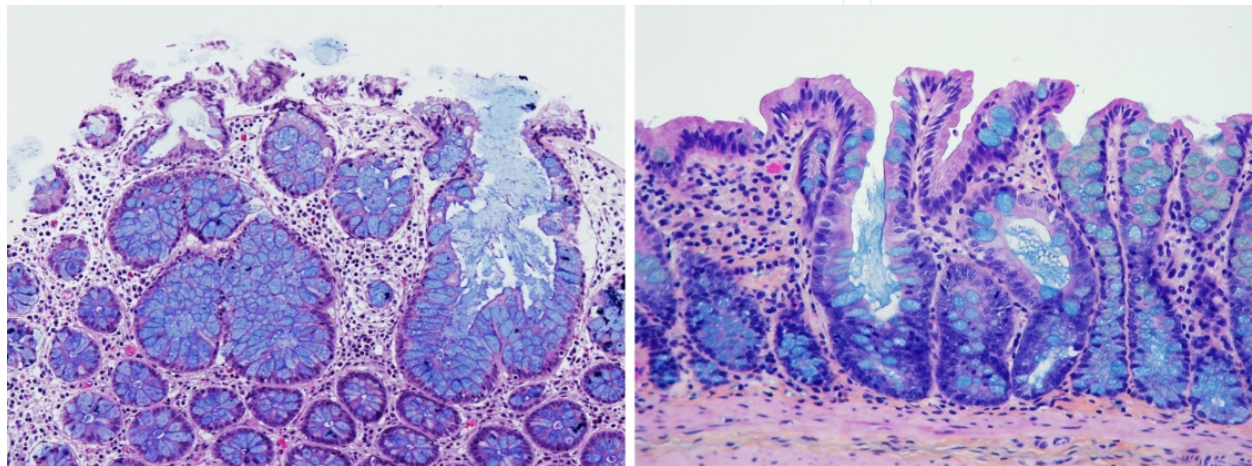


Figure 2. Hyperplastic aberrant crypts of human (left) colorectal mucosa accompanying resected sporadic colorectal adenoma. The focus is composed of 3 hyperplastic crypts that are much wider than surrounding normal crypts. The epithelial cells of the hyperplastic crypts are higher and composed of one layer. On the right there are hyperplastic aberrant crypts of rat colorectal mucosa, induced by carcinogen. The focus is composed of 3 crypts with slight mucin depletion. The level of focus is higher than of the surrounding mucosa. The epithelial cells of the hyperplastic crypts are higher and composed of one layer (Kreyberg trichrom stain).

Mitotic figures are limited to the lower two-thirds of the crypts and are never observed on the surface of crypts. Nuclei are basally located, ovoid or round, with occasional visible nucleoli and usually uniformly dark. The luminal opening of crypts is slightly elevated from the surrounding normal mucosa and the crypts are elongated and occasionally branching with partial mucin depletion [17]. The role of hyperplastic aberrant crypts in the process of colon carcinogenesis is not clear and is a matter of debate and further investigations [15].

5. ACF with dysplastic crypts (intraepithelial neoplasia/dysplasia)

Presence of dysplasia is regarded as early histopathological changes in the precursor lesions of colon cancer. The word dysplasia is histological term that describes structural and cytological alterations in the epithelium that predispose an organ to cancer development. Intraepithelial neoplasia (IEN) is a histological term for dysplastic lesions in the epithelial layer of colon mucosa that can be identified only after careful histological examination (Figure 3). IEN is

synonymous with terms atypical hyperplasia, microadenoma, carcinoma *in situ* and dysplasia. Depending on cytological and architectural features IEN is classified as low-grade or high grade. The differential histological criteria involve hypercellularity with enlarged, hyperchromatic nuclei, varying degrees of nuclear stratification, loss of polarity, high nuclear/cytoplasmic ratio, nuclear crowding, increased mitotic index and decreased mucin excretion [17; 20].

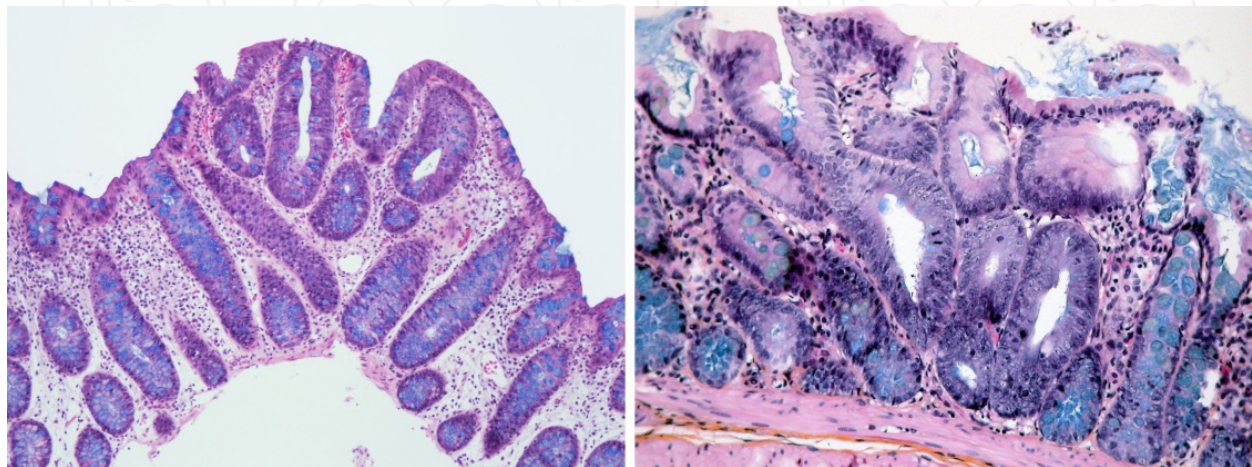


Figure 3. Dysplastic aberrant crypts of human colorectal mucosa accompanying FAP (left). The focus is composed of 4 crypts. The level of focus is higher than that of the surrounding mucosa. The epithelial cells covering the aberrant crypts are classified as mild dysplasia. On the right there are dysplastic aberrant crypts of rat colorectal mucosa induced by carcinogen. The focus is composed of 3 crypts that show severe mucin depletion. Numerous mitoses, stratification of nuclei, atypical epithelial cells, and architectural atypia are the components of dysplasia (Kreyberg trichrom stain).

6. Molecular characteristics of ACF

Evidence from experimental and clinical studies demonstrates that ACF share similar histological and molecular features as colonic tumors (i.e. adenomas and adenocarcinomas) [15; 21; 22]. Today, high-magnification chromoscopic colonoscopy allows detection and biopsy of ACF *in vivo* in man. It also provides opportunity to investigate and characterize the earliest genetic and molecular alterations in CRC development in man. ACF exhibit many of the molecular and genetic abnormalities that form the basis for the adenoma-carcinoma sequence in CRC.

It has been found that ACF exhibit many of the molecular and genetic abnormalities that form the basis for the adenoma-carcinoma sequence in CRC. Genetic alterations found in human ACF include mutations in tumor suppressor genes, microsatellite instability, aberrant methylation as well as aberrant expression of proteins (summarized in Figure 4). Up to date, three molecular pathways of CRC development have been identified and described, i.e. chromosomal instability, microsatellite instability and CpG island methylator phenotype. All three types of molecular alterations have also been found in ACF [22; 23].

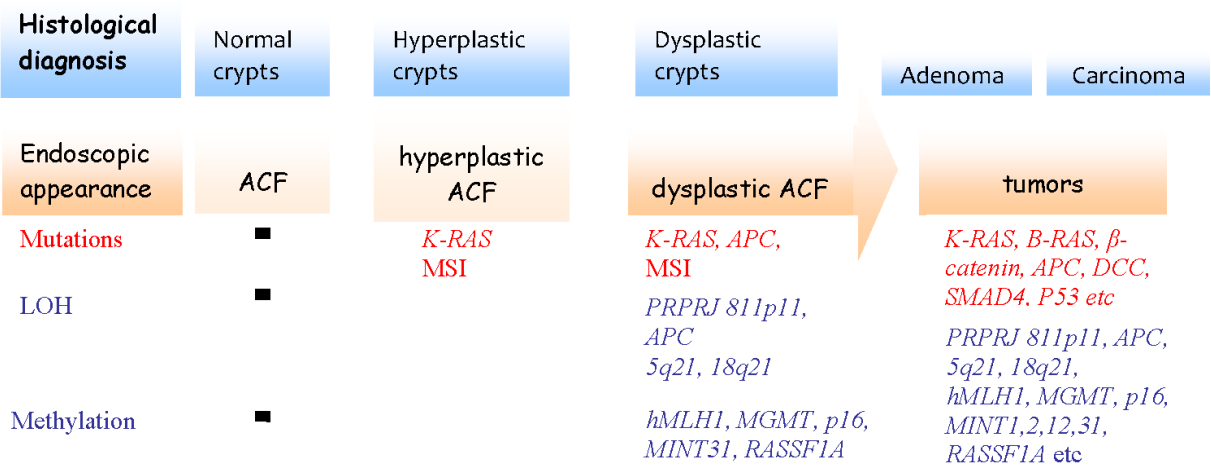


Figure 4. Phenotypic, genetic and epigenetic alteration involved in multistep development of colon carcinogenesis.

7. The chromosomal instability (CIN)

CIN is the most common in sporadic CRC and shows chromosomal abnormalities such as chromosome breaks, duplication, rearrangements, loss of heterozygosity (LOH) and sequential inactivation of tumor suppressor genes such as APC (5q), P53 (17p) and SMAD4 (18q), which are frequently found in sporadic carcinomas. It is known that germ line mutations in APC lead to the hereditary syndrome of FAP [22; 23].

Loss of heterozygosity (LOH) was observed in ACF at 18q (locus that maps close to the DCC and DPC4 genes) [24], in 67% LOH was found at locus 11p11, the location of the gene for protein tyrosine phosphatase receptor type J (PTPRJ; tumor suppressor gene), at locus 5q21 and 18q21 [25]. LOH was identified near the APC tumor suppressor gene (at the D5S346 marker) [26].

APC mutation was found in ACF with dysplastic crypts but not in ACF with hyperplastic crypts. This was frequently observed in ACF obtained from patients with FAP, while in ACF obtained from patients with sporadic CRC APC mutation was rarely observed [7; 27; 28].

β-catenin mutation, which is found in 12% of adenomas and 16% of carcinomas, was not found in ACF, regardless of the histologic type of ACF [5; 29]. Only increased expression of β-catenin was found in the cytosol of ACF with dysplastic crypts (54%) [30; 31].

K-RAS mutation was found in 13%-95% of ACF and was much more frequent in ACF with hyperplastic crypts (80%-100%) than in ACF with dysplastic crypts (0%-57%) [7; 27; 28; 32-35].

8. The microsatellite instability (MSI)

MSI is a hallmark of defective DNA mismatch repair (MMR) genes such as *hMLH1* or *hMSH2* and leads to the accumulation of a high frequency of somatic mutations [24]. It is frequent in patients with HNPCC (90%) but also found in sporadic CRC (15%).

MSI was identified in 30% ACF from patients of elevated risk (family or personal history) and 13% lesions from average risk patients (subjects without family or personal history) [24-26].

9. The CpG island methylator phenotype (CIMP)

CIMP is epigenetic mechanism characterized by hypermethylation of cytosine residue within CpG islands in the promoter regions of certain genes (which are particularly rich in CpG nucleotides such as tumor suppressor and MMR genes) and results in their inactivation (loss of gene expression). Genes that have been shown to be silenced by promoter hypermethylation in human CRC are *p16*, *hMLH1*, *MGMT*, *MINT1,2,12*, and *31* [22; 23].

Methylation of CpG islands was found in 34% ACF obtained from patients with both FAP and sporadic CRC, but was more frequent in sporadic ACF (53%), especially dysplastic ACF (75%) and less hyperplastic ACF (7%) [32].

In ACF hypermethylation of the MMR genes such as *hMLH1* and *MGMT* (in hyperplastic ACF) [26] and tumor suppressor genes such as *p16*, *MINT31* [32], *RASSF1A* were found [26].

10. ACF revealing new insight into CRC development

As high-magnification chromoscopic colonoscopy now allow detection and biopsy of ACF in the mucosa of large bowel, ACF might serve as a research tool for revealing new insights and knowledge into first alterations and risk factors in CRC development.

Evidence shows that ACF in FAP patients differ from ACF in subjects with sporadic CRC. Differences can be observed regarding endoscopic, histological and molecular characteristics. Patients with FAP have significantly increased number of ACF than patients with sporadic CRC. Most of the ACF in FAP patients is histologically diagnosed as dysplastic (89%), while patients with sporadic CRC have mostly ACF with hyperplastic crypts (82%). *K-RAS* mutation was found very rarely in dysplastic ACF of FAP patients, but frequently in ACF obtained from patients with sporadic CRC (82%) [7].

Conversely, *APC* mutations were very rarely found in dysplastic ACF from patients with sporadic CRC, while in ACF from FAP patients were almost always present. Methylation of CpG islands was found in sporadic ACF but not in ACF from FAP patients [32].

Differences in the endoscopic appearance and genetic features were observed also in ACF obtained from patients with ulcerative colitis (UC). In ACF from patients with UC *K-RAS*

mutation was rarely observed and *APC* mutation was not found. However, in dysplastic ACF frequent methylation of promoter region of *p16* (73%) and *P53* mutation (60%) was found [36].

All these data show that ACF provide opportunity to get closer insight into first molecular events that are responsible for initiation and formation of CRC.

11. Endoscopic characteristics of ACF

As already mentioned ACF are the first lesions that can be found on the surface of the fixed or fresh colorectal mucosa. ACF are invisible to standard endoscopic instruments but can now be visualized *in vivo* endoscopically by using specialized magnifying colonoscopes in conjunction with dye sprays (methylene blue, indigo carmine), the technique termed high-magnification chromoscopic colonoscopy [6].

Staining of the colonic mucosa at colonoscopy improves the visibility of the morphological characteristics of the crypts in the mucosa, such as shape, size or luminal openings of the crypts. Most prominent feature of aberrant crypts is that they stain more darkly than do normal surrounding crypts. However, there are also other morphological features important for identification of ACF. By definition, ACF are colon crypts that are larger than normal surrounding crypts, have increased pericryptal space that separates them from the normal crypts, they have a thicker layer of epithelial cells that often stains darker, their luminal openings are not circular but rather oval or even compressed. They are usually not in the same level as the surrounding normal crypts but they are either slightly elevated above the mucosa or may be even depressed. ACF may be composed of one to few hundreds of aberrant crypts per focus (1 to 412) [10; 14; 15].

As explained, ACF are heterogeneous group of lesions that exhibit variability in histological and molecular characteristics as well as variability in morphological characteristics.

Based on the surface morphologic features of ACF, researchers are able to distinguish three types of ACF and predict histologic characteristics of ACF [6].

Aberrant crypts that stain more darkly and are larger, have a thicker epithelial lining and a larger pericryptal zone than normal crypts and exhibit large oval (smooth, dilated) lumens have been histologically diagnosed as almost normal. Such ACF have slight enlargement, irregularity, and elongation of the ducts but show no signs of hyperplasia or dysplasia [6].

Aberrant crypts that have all above-mentioned characteristics and exhibit asteroid or slit shape of lumens have been histologically diagnosed as hyperplastic with serrated luminal pattern. Aberrant crypts that have thicker epithelial lining than both above mentioned types and exhibit compressed or undistinguishable lumen are classified in the third group of ACF, histologically diagnosed as dysplastic. Such ACF show loss of polarity, hyperchromatism and stratification of the nuclei in the crypt epithelium [6; 12; 14].

12. Prevalence and density of ACF

First data about density (average number of ACF per cm² of mucosa) and anatomical location of ACF in colonic mucosa are based on investigations of colorectal resections. Results have shown that patients with increased risk (personal or family history) have higher average number of ACF per cm² than persons with average risk (subjects without personal or family history). It was found that FAP patients have significantly higher density of ACF in colon mucosa than patients with sporadic CRC or benign bowel disease. Higher frequencies of ACF were observed in left than in right colon. Results have shown that density of ACF increases from proximal to distal part of the colon, being the highest in the rectosigmoidal region, which corresponds to anatomical location of CRC development [11-14; 34].

13. Endoscopic detection of ACF

Similar findings regarding prevalence and distribution of ACF in colorectal mucosa have been observed in humans *in vivo* by using magnifying (40x) endoscopy after endoscopic staining of colon mucosa (methylene blue). Takayama et al. [6] examined 370 subjects (147 normal subjects, 130 patients with adenoma, and 48 patients with carcinoma) and found that ACF were present in almost all patients with adenoma or carcinoma. ACF were most frequently observed in the left colon, where polyps are often found. Additionally, it was found that patients with adenoma or carcinoma had significantly higher estimated relative risks for ACF with dysplastic crypts than normal subjects [6].

In normal subjects, both the prevalence and the number of ACF in subjects under 40 years of age were very low (10%) but increased with age, particularly after the age of 40 (54% - 66%). Conversely, patients with cancer had a consistently high prevalence (100%) and large number of ACF regardless of age. In patients with adenoma, the age-associated increment in the prevalence and number of ACF was intermediate [6].

Takayama et al. [6] investigated number, density, and dysplastic features of distal colorectal ACF in patients with exophytic adenomas and carcinomas, while Hurlstone et al. [16] assessed the prevalence and features of ACF in patients with flat and depressed colorectal neoplastic lesions, which account for around one third of all colorectal lesions. High magnification chromoscopic colonoscopy was performed on 574 healthy subjects, 281 patients with flat adenomas and 14 patients with flat carcinomas in which 602 (3% of them dysplastic), 2796 (18% dysplastic) and 594 (61 % dysplastic) ACF were identified, respectively. Similarly as in patients with exophytic colorectal lesions, the number of ACF increased in a stepwise fashion from normal subjects to patients with flat or depressed adenoma and then to patients with flat or depressed carcinoma [16].

In another study 103 patients with average age of 61 (range of 28-87) were examined by using magnification (60x) chromoscopic colonoscopy. 788 ACF were found in the distal 20 cm of colon/rectum. Patients with a family history of CRC had a significantly higher mean number of ACF than the average risk subjects (7.6% dysplastic and 46% hyperplastic) [5].

Rudolph et al. [37] have demonstrated that the number of ACF is significantly increased in patients with personal history of adenoma in comparison to subjects without personal or family history. They also observed that number of ACF is higher in older persons than in younger subjects [37].

14. Clinical application

Clinical application of ACF as an intermediate biomarker for CRC in humans is under development and is thus less conclusive [4; 38].

Recently, few studies investigated relationship of human colorectal ACF and formation of colorectal polyps on repeat colonoscopy. It was found that the number of ACF in the colorectum was associated with substantial risk for future advanced neoplasia [39-42].

Ohkubo et al. [39] investigated natural history of human ACF and correlation with risk factors for CRC. They examined 82 subjects who underwent total colonoscopy and whose ACF number was examined at least 2 times. They retrospectively evaluated the changes in the ACF number at four different surveillance periods (6 months, 1 year, 2 years, 3 years) and in groups with and without colorectal neoplasms. The subjects were classified into an increased ACF group and a no change/decreased ACF group, and investigated the relationship between the changes in the ACF number and known risk factors for CRC. No significant differences were observed in the ACF number between the first and second observations in any surveillance period groups, and in the groups classified according to the presence or absence of colorectal neoplasms. There were no significant differences between the increased and no change/decreased ACF groups in terms of gender, smoking habit, current alcohol consumption, age, BMI, HbA1c or serum triglyceride level, whereas a significant difference between the groups was observed in the serum total cholesterol level [39].

All these data strongly implicate that detection and quantification of ACF in the distal colon may be useful in predicting CRC risk and may be considered as a useful marker in chemopreventive trials. Furthermore, it is expected that one of the most important clinical applications of ACF observation with magnifying endoscopy will be its use as a target lesion for chemoprevention. Because ACF are small lesions, they are suggested to be eradicated during a short time by administration of chemopreventive agents [43]. Takayama et al. [43] performed an open chemopreventive trial of sulindac and found that the number of ACF was reduced markedly in 2 months. Patients receiving sulindac for more than one year had no ACF in colon mucosa. After 8 to 12 months of follow-up, the number of ACF in colorectal mucosa significantly decreased or even completely disappeared. In the untreated control subjects the number of ACF was either unchanged or slightly increased [6]. Another short-term chemoprevention trial of metformin for colorectal ACF showed suppressive effect of the drug on the formation of ACF [44]. Other chemopreventive a double blind randomized controlled trial targeting ACF are under investigations [43-45].

15. Difficulties or pitfalls in detection of ACF

All these data strongly suggest that ACF in the distal colon may be useful and reliable surrogate marker in predicting CRC risk. However, there are also limitations and difficulties. The main limitation is the fact that chromoendoscopy and magnifying endoscopes are largely research tools and not the equipment in gastrointestinal practice. Difficulties were reported in some studies in which endoscopic criteria failed to predict histologic confirmation of ACF or correlation between the number of ACF and CRC risk [46; 47]. It was also found that there was considerable variability among endoscopists regarding accuracy to correctly identify ACF. It was found that in spite of training, accuracy to correctly identify ACF did not improve [46].

Current knowledge about rodents ACF, which share many similarities with human pathology, might be helpful to understand tricks and traps when using ACF. In rodents, ACF are widely accepted as intermediate biomarkers of CRC risk assessment. They have been used as an endpoint in identifying and assessing preventive or promotional role of natural and pharmacological compounds, as well as dietary and environmental factors in the process of colon carcinogenesis [48; 49]. However, limitations to the use of ACF as a biomarker to identify cancer preventive agents exist. Increasing number of studies has demonstrated that ACF in both animals and humans are heterogeneous group of lesions that contain multiple genetic, epigenetic and phenotypic alterations [15; 22; 50]. In rodents, total number of ACF may be considered as a valid biomarker only at very early stage of carcinogenesis, while in subsequent weeks ACF with higher crypt multiplicities (more than 4 crypts) are considered more specific biomarker than total number of ACF. In more advanced stages of colon carcinogenesis ACF may not be reliable intermediate biomarker of colon carcinogenesis (explained in detail in [9] and [51]). It is also important to mention that ACF are not equally distributed among the proximal, middle or distal colon. The majority of ACF develop in the middle and distal colon [52-54], which need to be taken into consideration when using ACF as biomarkers (comprehensively discussed in [9; 10] and [51]. Nevertheless, when considering all above mentioned facts ACF are useful biomarkers for the screening of compounds for their chemopreventive activities [49; 51].

16. Conclusion

Based on experimental and clinical studies evidence demonstrates that ACF share similar histological and molecular features as colonic tumors (i.e. adenomas and adenocarcinomas). ACF exhibit many of the molecular and genetic abnormalities that form the basis for the adenoma-carcinoma sequence in CRC. Today, high-magnification chromoscopic colonoscopy allows detection and biopsy of ACF *in vivo* in man. It also provides opportunity to investigate and characterize the earliest genetic and molecular alterations in CRC development in man.

However, it has been shown that ACF are heterogeneous group of lesions that exhibit variable endoscopic, histological and molecular features. This fact has been shown to cause some difficulties in accuracy of detection and quantification of ACF among endoscopists. However,

chromoendoscopy and magnifying endoscopes are largely research tools and future research on that field will bring new information about reliability and applicability of ACF as biomarker of CRC risk in clinical practice.

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