Aberrant Crypt Foci as Precursors of the Dysplasia-Carcinoma Sequence in Patients with Ulcerative Colitis

Takehiro Kukitsu, Tetsuji Takayama, Koji Miyanishi, Atsushi Nobuoka, Shinichi Katsuki, Yasushi Sato, Rishu Takimoto, Takuya Matsunaga, Junji Kato, Tomoko Sonoda, Sumio Sakamaki, and Yoshiro Niitsu

Abstract

Purpose: Long-standing ulcerative colitis (UC) predisposes patients to the development of colorectal cancer, but surveillance of colitis-associated cancer by detecting the precancerous lesion dysplasia is often difficult because of its rare occurrence and normal-looking appearance. In sporadic colorectal cancer, aberrant crypt foci (ACF) have been reported by many investigators to be precursor lesions of the adenoma-carcinoma sequence. In the present study, we analyzed the genetic background of ACF to determine whether they could be precursors for dysplasia, and we examined the usefulness of endoscopic examination of ACF as a surrogate marker for surveillance of colitis-associated cancer.

Experimental Design: ACF were examined in 28 UC patients (19 patients with UC alone and 9 patients with UC and dysplasia; 2 of those patients with dysplasia also had cancer) using magnifying endoscopy. K-ras, APC, and p53 mutations were analyzed by two-step PCR RFLP, in vitro – synthesized protein assay, and single-strand conformation polymorphism, respectively. Methylation of p16 was analyzed by methylation-specific PCR.

Results: ACF that appeared distinct endoscopically and histologically were identified in 27 out of 28 UC patients. They were negative for K-ras, APC, and p53 mutations but were frequently positive for p16 methylation (8 of 11; 73%). In dysplasia, K-ras and APC mutations were negative but p53 mutation (3 of 5; 60%) and p16 methylation (3 of 5; 60%) were positive. There was a significant stepwise increase in the number of ACF from patients with UC alone to patients with dysplasia and to patients with cancer. Univariate and multivariate analyses showed significant correlations between ACF and dysplasia.

Conclusions: We have disclosed an ACF-dysplasia-cancer sequence in colitis-associated carcinogenesis similar to the ACF-adenoma-carcinoma sequence in sporadic colon carcinogenesis. This study suggests the use of ACF instead of dysplasia for the surveillance of colitis cancer and warrants further evaluation of ACF as a surveillance marker in large-scale studies.

It is commonly recognized that long-standing ulcerative colitis (UC) predisposes patients to the development of colorectal cancer (1). However, the detection of early colorectal cancer is often difficult in patients with UC because there is inflammation in the background mucosa and it predominantly represents flat-type ill-delineated lesions (2, 3). Therefore, colitis-associated cancer is often detected at an advanced stage and is characterized by a very poor prognosis. One approach to overcome this difficulty is to use dysplasia, which is considered to be a precancerous lesion in colitis-associated cancer, as a surrogate marker for early detection of colitis-associated cancer (4, 5). However, identification of dysplasia by endoscopy requires greater skill than detection of cancer itself because of its rare occurrence and apparently normal-looking appearance (6).

We previously succeeded in identifying aberrant crypt foci (ACF) in non-UC subjects using magnifying endoscopy (7) and showed that the number of ACF increased in the order of normal subjects, patients with adenomas and then patients with cancer, and proposed an ACF-adenoma-carcinoma sequence for sporadic colon carcinogenesis. Adler et al. also observed ACF using magnifying endoscopy and found that the number of rectal ACF in patients with colorectal cancer was significantly higher than in normal subjects (8). The increased number of ACF was further observed in patients with flat adenoma and cancer (9). Seike et al. showed that ACF could be a predictive factor for advanced rectal cancer by multivariate analysis (10). Thus, magnifying endoscopy has become a
common methodology to observe ACF. Regarding the genetic abnormality of ACF, we found a highly frequent K-ras mutation and GSTP1-1 overexpression and also showed that GSTP1-1 endows ACF with resistance to bile salt–induced apoptosis (11–13).

With regard to the genetic abnormality of colitis-associated cancer and dysplasia, mutations of K-ras and APC are relatively rare (14–17), and in contrast, p53 mutation is frequently positive (16, 18, 19). Moreover, hypermethylation of genes such as the p16 gene has recently been detected (20, 21). On the basis of these previous reports, we first attempted to define ACF as precursors for colitis-associated dysplasia by analyzing their genetic background, including mutation of K-ras, APC, p53, and hypermethylation of p16, and then examined the feasibility of using ACF as surrogate markers for the surveillance of colitis-associated cancer.

**Materials and Methods**

**Subjects.** This study was approved by the ethics committee of Sapporo Medical University. Fifty-six subjects were enrolled after obtaining written informed consent. The subjects were comprised of 28 UC patients (19 patients with UC alone and 9 patients with UC and dysplasia; 2 of those patients with dysplasia also had cancer), 24 healthy subjects, and 4 patients with Crohn’s disease as a control. Average ages and male/female ratios in these groups were as follows: UC: 38.3 ± 6.7 years and 1/1; healthy subjects: 41.5 ± 7.8 years and 3/5; Crohn’s disease patients: 30.3 ± 7.4 years and 2/2. The diagnosis of UC was made according to established criteria, including clinical symptoms, radiological findings, blood examination, endoscopy, and pathologic observation of inflamed intestinal mucosa.

**Magnifying endoscopy.** UC patients in remission underwent magnifying endoscopy, which was done as described previously (7, 11). In order to improve the visualization of ACF (i.e., accurate evaluation of ACF number), plenty of polyethylene glycol was administered before examination. All patients underwent total colonoscopy. After observation of the entire colorectum, the lower rectum from the middle Houston valve to the dentate line was washed with plenty of water, stained with 0.2% methylene blue, and again washed with sufficient water for identification of ACF. Biopsies were taken under magnifying endoscopy as described previously (7, 11). In this particular study, to avoid laborious and lengthy procedures considering the future application of ACF as a surveillance marker, the observation of ACF was limited to the lower rectum on the basis of our previous report; the number of ACF in the lower rectum correlated with that in the entire colorectum (7). All procedures were recorded on videotape and evaluated by two independent observers who were unaware of the subjects’ clinical histories.

**ACF criteria.** ACF were defined as lesions in which crypts were more darkly stained with methylene blue than normal ones and had larger diameters, often with oval or slit-like lumens and thicker epithelial linings (22, 23).

**Two-step PCR and RFLP for detection of K-ras codon 12 mutations.** Cellular DNA was extracted from the biopsy specimens and used as a template for PCR. The PCR products were amplified using mismatched primers and analyzed by RFLP to detect point mutations in codon 12 of the K-ras gene, as described previously (12, 24).

**In vitro–synthesized protein assay for detection of mutations in APC.** In vitro–synthesized protein assays were performed according to a method described previously (25). In brief, primer pairs were prepared for segments 3 (codons 686-1022) and 4 (codons 996-1693) of the APC gene, which include the whole mutation cluster region. These primer pairs were specially designed to place the necessary transcriptional and translational regulatory sequences at the 5′-ends of the PCR products. Genomic DNA were extracted from ACF and dysplasia tissue samples were obtained by microdissection. After amplification of the APC gene, the PCR products were used directly, without cloning, as templates in coupled transcription and translation reactions (Promega Corp.) in a mixture containing 10 μCi of 35S-methionine. The proteins thus synthesized were analyzed on 10% to 20% gradient SDS polyacrylamide gels and visualized by autoradiography.

**Single-strand conformation polymorphism analysis of p53.** Four primer pairs for exons 5, 6, 7, and 8/9 (Takara), which include the hotspot region of p53 mutation were used. Single-strand conformation polymorphism analyses were done according to the method described previously (26). In brief, genomic DNA were amplified using each primer pair. Aliquots of the PCR product were denatured for 5 min at 80°C in a sample buffer containing 98% formamide, and then cooled quickly on ice. Each sample was electrophoresed on a 15% polyacrylamide gel, which was stained using a silver staining kit (Bio-Rad).

**Analysis of hypermethylation of p16.** Bisulfite treatment of DNA was done as described previously (27). Briefly, 2 μg of genomic DNA were denatured in 0.2 mol/L of NaOH at 37°C for 20 min, followed by incubation with 3 mol/L of sodium bisulfite (Sigma Chemical Co.). Hydroquinone (Sigma Chemical Co.) was added at a final concentration of 0.5 mmol/L. The reaction was done at 55°C for 16 h. After treatment, modified DNA was purified using a Wizard DNA Clean-Up kit (Promega) as recommended by the manufacturer, and resuspended in 30 μL of distilled water. Two microliters of the bisulfite-incubated DNA were used as a template for each bisulfite-PCR, and primer pairs were used as described previously (27). After amplification, each PCR sample was electrophoresed on 10% to 20% polyacrylamide gels, stained with ethidium bromide and directly visualized under UV illumination.

**RNA extraction and reverse transcription-PCR.** Total RNA was isolated from the frozen samples of ACF and normal mucosa of UC patients using the total RNA isolation system (Promega). Reverse transcription-PCR (RT-PCR) was done as previously described (28). Briefly, the reverse transcription reactions were achieved using avian myeloblastosis virus reverse transriptases. Then, the p16INK4A-specific exon 1 was amplified with primers 5′-ATGGACCTTCCGCTGACGTG-3′ and 5′-GATCGCCCTCGACGGCTTAC-3′. Glycerinaldehyde-3-phosphate dehydrogenase was used as an internal standard.

**Statistical analysis.** The number of ACF in relation to potential risk factors for colitis-associated cancer were compared by Mann-Whitney U test. Multivariate analysis was carried out by multiple logistic regression analysis using SAS software (SAS Institute Japan).

**Results**

**Endoscopic appearance and histology of ACF in UC patients.** An endoscopic view of dysplasia, which is often difficult to identify by standard endoscopy in a patient with UC is shown in Fig. 1A. Histologically, the nuclei of goblet cells are hyperchromatic, have lost their normal polarity, and show some nuclear stratification. These characteristics are consistent with low-grade dysplasia (29). Figure 1D shows a representative endoscopic view of ACF in a patient with UC (colitis ACF) in comparison with typical ACF in non-UC patients (non-UC ACF), which we reported previously (Fig. 1G). Both types of ACF could be identified as a focus consisting of large crypts darkly stained with methylene blue. However, in comparison to the typical non-UC ACF, the lining of each crypt of the colitis ACF was obscure and the boundaries of individual crypts were unclear. Moreover, most of the colitis ACF showed distorted shapes in contrast to round or oval shapes of the non-UC ACF in the majority. Histologically, the
Colitis ACF specimens showed marked infiltration of lymphocytes in the stroma (Fig. 1E) as compared with non-UC ACF (Fig. 1H). They also showed a more diverse range of crypt sizes, enlarged nuclei in epithelial cells, and increased chromatin staining.

We identified a total of 164 ACF in 27 out of 28 patients with UC by using methylene blue in magnifying endoscopy, and no side effects were noted. Of these, 147 (91.3%) showed the typical appearance of colitic ACF as illustrated in Fig. 1D. Only 17 ACF (9.7%) in these patients showed the typical appearance of non-UC ACF. Therefore, we confined further examinations to the colitis ACF.

Colitis-associated colorectal cancer is reported to have a higher incidence of histologically mucinous type tumors than sporadic colorectal cancer. It has also been reported that goblet cell hyperplasia is frequently observed in dysplasia from patients with UC and that the frequency of goblet cell hyperplasia is positively correlated with the duration of UC (30). Therefore, goblet cells in nine colitis ACF, nine non-UC ACF, seven dysplasia, and seven normal rectal epithelial specimens were examined by Alcian blue staining. The frequencies of goblet cells observed in colitis ACF and dysplasia tissues were 49.5 ± 9.7% and 52.7 ± 14.2%, respectively. They were significantly greater than those in the normal background mucosa of ACF tissues (32.3 ± 5.9%) and in non-UC ACF (35.7 ± 15.4%), suggesting the existence of goblet cell hyperplasia in colitis ACF as well as dysplasia. These results are also consistent with the hypothesis that ACF are precursor lesions of dysplasia in patients with UC.

**Analysis of K-ras mutation in colitis ACF and dysplasia.**
Because K-ras mutations have frequently been detected in ACF from non-UC patients (11, 31, 32), we screened colitis ACF specimens and dysplasia tissue from UC patients for K-ras codon 12 mutations by a two-step PCR-RFLP method. K-ras mutations were found in 20% (2 of 10) and 0% (0 of 5) of colitis ACF and dysplasia specimens, respectively, from UC patients.

**Fig. 1.** Endoscopic and histologic features of dysplasia (A-C), ACF in a patient with UC (D-F), and ACF in a non-UC patient (G-I). A, dysplasia in a UC patient, which was not visible unless chromoendoscopy was done. B, the crypt was lined by columnar epithelia with some nuclear stratification, hyperchromatic nuclei, and loss of normal polarity (H&E; magnification, ×150). C, increased numbers of goblet cells were seen in the dysplasia of UC patients. Some dystrophic goblet cells were observed (Alcian blue; magnification, ×150). D, a representative ACF in a UC patient, which was characterized by darker staining with methylene blue and larger crypts with thicker epithelial lining than the background mucosa. The lining of each crypt was obscure and the boundaries of individual crypts were more unclear than in non-UC ACF (G). E, the colitis ACF showed marked infiltration of lymphocytes in the stroma, a more diverse range of crypt sizes, enlarged nuclei in epithelial cells, and increased chromatin staining compared with non-UC ACF (H&E; magnification, ×120). F, an increase in the number of goblet cells was seen in colitis ACF, similar to dysplasia in UC patients. Some dystrophic goblet cells were also identified (Alcian blue; magnification, ×120). G, a representative non-UC ACF consisting of crypts with round and oval lumens and with a wide pericryptal space. H, non-UC ACF showed slight enlargement, irregularity, and elongation of the ducts (H&E; magnification, ×150). I, the number of goblet cells in non-UC ACF was apparently fewer than that in colitis ACF (Alcian blue; magnification, ×150).
patients. In contrast, mutations were detected in 4 of 5 (80%) non-UC ACF specimens (Fig. 2), consistent with our previous reports and those of other laboratories (7, 11, 14, 15). Thus, the frequency of K-ras mutations in colitis ACF was relatively low compared with that of non-UC ACF.

Analysis of APC mutation in colitis ACF and dysplasia. Because APC mutation is an early genetic event in colorectal carcinogenesis in non-UC patients (32–34), we examined APC mutations in colitis ACF and dysplasia specimens. Segments 3 and 4 of the APC gene, which include the entire mutation cluster region, were analyzed by an in vitro -synthesized protein assay in 11 colitis ACF and 2 dysplasia tissue specimens. No APC mutations were detected in any of the 11 colitis ACF (0 of 11, 0%) or the 2 dysplasia specimens (0 of 2, 0%; Fig. 3). Likewise, no APC mutations were detected in any of the 7 ACF specimens from non-UC patients (data not shown), consistent with our previous report (11).

Analysis of p53 mutation in colitis ACF and dysplasia. It has been reported that p53 mutations are frequently detected in dysplasia and cancer tissues from patients with UC (16, 18, 19). Therefore, we investigated for p53 mutations in the hotspot region (exons 5-9) employing nonradioisotopic single-strand conformation polymorphism in 11 colitis ACF and 5 dysplasia specimens from patients with UC. No mutations were detected in any of the exons 5, 6, 7, or 8/9 in the 11 ACF specimens, whereas mutations were detected in exons 5, 6, or 8/9 of the 5 dysplasia specimens (Fig. 4). Overall, for exons 5 to 9, p53 mutations were found in 3 of the 5 dysplasia specimens (60%) but in none of the 11 ACF lesions (0%) from patients with UC.

Hypermethylation of the p16 gene promoter in colitis ACF and dysplasia. Recently, it was reported that the p16 gene promoter is often hypermethylated in dysplasia and cancer from UC patients (20, 21). Therefore, we examined the methylation status of the promoter region of the p16 gene in 11 ACF, 5 dysplasia, and 4 normal epithelia specimens from 4 UC patients using methylation-specific PCR. No methylation of the p16 gene promoter, which was represented by a 152 bp band, was detected in any of the 4 normal epithelia specimens. However, it was found in 8 of the 11 ACF (73%) and in 3 of the 5 dysplasia specimens (60%; Fig. 5A).

We then determined the expression of p16\textsuperscript{INK4A} mRNA employing RT-PCR in eight ACF, two dysplasia, and four normal epithelia specimens from the other four UC patient groups. The reason we dealt with specimens from other UC groups (four patients) than the group (four patients) for methylation analyses, was that analyses of methylation and mRNA on the same small specimens was technically difficult. Nevertheless, the p16\textsuperscript{INK4A} mRNA was readily detected in all four specimens of normal epithelia. Although it was detectable only in two of eight (25%), very faintly detectable in one of eight (12.5%), and undetectable in five of eight (63%) ACF specimens and was undetectable in two of two dysplasia specimens (Fig. 5B). These results suggested that p16\textsuperscript{INK4A} expression is suppressed by the methylation of its promoter.

The number of colitis ACF in UC patients with or without dysplasia. If ACF are indeed precursor lesions of dysplasia, it would be expected that UC patients with dysplasia would have more ACF than those without dysplasia. Therefore, we investigated the number of ACF in UC patients with and without dysplasia and compared them. The number of ACF in the dysplasia-positive group (8.7 ± 4.5) was significantly higher than that in the dysplasia-negative group (3.5 ± 2.6; P = 0.0112). In particular, the number of ACF in the two patients with both dysplasia and cancer were 17 and 13, respectively, which represents very high numbers even for the dysplasia-positive group. All cases in the dysplasia-positive
group had five or more ACF. The mean number of ACF in age-matched healthy subjects (1.0 ± 1.7) was apparently smaller than that in either the dysplasia-positive or dysplasia-negative group of UC patients. The mean number of ACF in the four patients with Crohn’s disease, an inflammatory bowel disease from which cancer develops at a low rate, was only 0.3 ± 0.6 (Fig. 6).

The number of colitis ACF in relation to potential risk factors for colitis cancer. We analyzed the relationship between the number of ACF and potential risk factors for colitis cancer such as gender, age at onset, the extent of lesions, duration of disease, and the existence of dysplasia. Univariate analyses showed significant correlations between ACF numbers and the existence of dysplasia ($P = 0.0112$) or duration of disease ($P = 0.0306$). There were no significant correlations between the number of ACF and gender, age, or extent of lesions (Table 1). Multiple logistic regression analysis of the relationship between dysplasia and various background factors showed significant correlations between the presence of dysplasia and the number of ACF ($P = 0.0189$) or disease duration ($P = 0.0492$; Table 2).

Discussion

In this study, we successfully and to our knowledge, for the first time, identified colitis ACF employing magnifying
endoscopy, which were more darkly stained with methylene blue than normal crypts and had larger diameters with oval or slit-like lumens and thicker epithelial linings (7, 11). The appearance of colitis ACF was distinct from sporadic ACF. Endoscopically, the boundaries of individual crypts in colitis ACF were obscure in contrast to the clear lining of each crypt in sporadic ACF, and most of the colitis ACF showed distorted shapes in contrast to round or oval shapes of sporadic ACF. Histologically, much more lymphocyte infiltration was seen in colitis ACF than in sporadic ACF.

Differences were also evident with respect to genetic background. Sporadic ACF were frequently positive for K-ras mutation and p16 overexpression, as previously reported by us (36–38). In contrast, colitis ACF were negative for K-ras mutation and p16 expression, whereas colitis ACF were essentially negative for K-ras mutation and also negative for p16 expression due to hypermethylation of the gene. These results suggest that the etiology of ACF may be different in UC patients from that in sporadic ACF subjects. In this context, it is intriguing that in other types of inflammation-associated carcinogenesis, such as hepatitis C–associated hepatocellular carcinoma and chronic gastritis-associated gastric cancer, silencing of the p16 gene (p16 hypermethylation) is frequently observed (36–38).

With regard to the relationship between colitis ACF and dysplasia, it is highly plausible that the former are precursor lesions of the latter because the gene abnormalities of both lesions were similar in terms of negativity for APC and K-ras and positivity for p16 hypermethylation. Multivariate analysis showed a close correlation between the number of ACF and occurrence of dysplasia, which also strongly supported the precursor theory of ACF. The close correlation between lesions and the fact that ACF were readily detectable in higher numbers than dysplasia, which requires total chroendoscopy spraying methylene blue on the entire colorectum for detection, suggests that ACF are a more appropriate surveillance marker than dysplasia for colitis-associated cancer.

Incidentally, the ACF found in UC patients were not all colitis ACF but were mixed with typical sporadic (non-UC) ACF, as far as endoscopic appearance was concerned. However, the incidence was very low (only 9.7%). This may be explained by the fact that the prevalence of sporadic ACF sharply increases after the age of 40 to 50 years (7), whereas the mean age of UC patients in this study was 38.3 ± 6.7 years. Nevertheless, because of the low incidence, sporadic ACF contamination with colitis ACF would not hamper the usefulness of colitis ACF as a surveillance marker.

A possible obstacle to using colitis ACF as a surveillance marker is that patients are obliged to undergo endoscopic examination, which itself may aggravate UC activity. However, this is unlikely to be a significant obstacle as long as endoscopy is done only when UC is in an inactive state and the survey of ACF is limited to the rectum, spending only 10 to 15 min on the whole procedure on the basis of our previous finding that the number of ACF in the rectum correlated with that in the entire colorectum (7). In this study, indeed, no particular aggravation of UC activity was observed in any of the patients with the evidential results of univariate and multivariate analyses supporting the validity of the use of rectal ACF in place of entire colorectal ACF as a surveillance marker.

In conclusion, in this study, we disclosed an ACF-dysplasia-cancer sequence in colitis-associated carcinogenesis similar to the ACF-adenoma-carcinoma sequence in sporadic colon carcinogenesis. We then proposed the feasibility of using ACF instead of dysplasia for the surveillance of colitis-associated cancer. Further evaluation of ACF as a surveillance marker in large-scale studies is warranted.

### Acknowledgments

The authors thank Dr. T. Okamoto at the Fourth Department of Internal Medicine, Sapporo Medical University School of Medicine for tissue collection.

### Table 1. Univariate association of ACF with potential risk factors for colorectal cancer in UC patients

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<th>Risk factor</th>
<th>Number of ACF-like lesions</th>
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<tr>
<td>Gender</td>
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<tr>
<td>Male</td>
<td>5.0 ± 3.9</td>
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<tr>
<td>Female</td>
<td>6.7 ± 4.9</td>
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<tr>
<td>Age at onset (y)</td>
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<tr>
<td>&lt;40</td>
<td>5.7 ± 4.7</td>
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<tr>
<td>≥40</td>
<td>6.0 ± 4.3</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
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<td>Total colon</td>
<td>7.0 ± 4.6</td>
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<tr>
<td>Left side colon</td>
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<td>Duration (y)</td>
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<tr>
<td>&lt;8</td>
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</tr>
<tr>
<td>≥8</td>
<td>7.3 ± 4.9</td>
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<td>Dysplasia</td>
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<tr>
<td>Positive</td>
<td>8.7 ± 4.5</td>
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<tr>
<td>Negative</td>
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### Table 2. Multiple logistic regression analysis of risk factors associated with dysplasia in patients with UC

<table>
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<th>Risk factor</th>
<th>Odds ratio (95% confidence interval)</th>
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<td>Gender</td>
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<td>Age</td>
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<td>Duration</td>
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<td>ACF</td>
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References
