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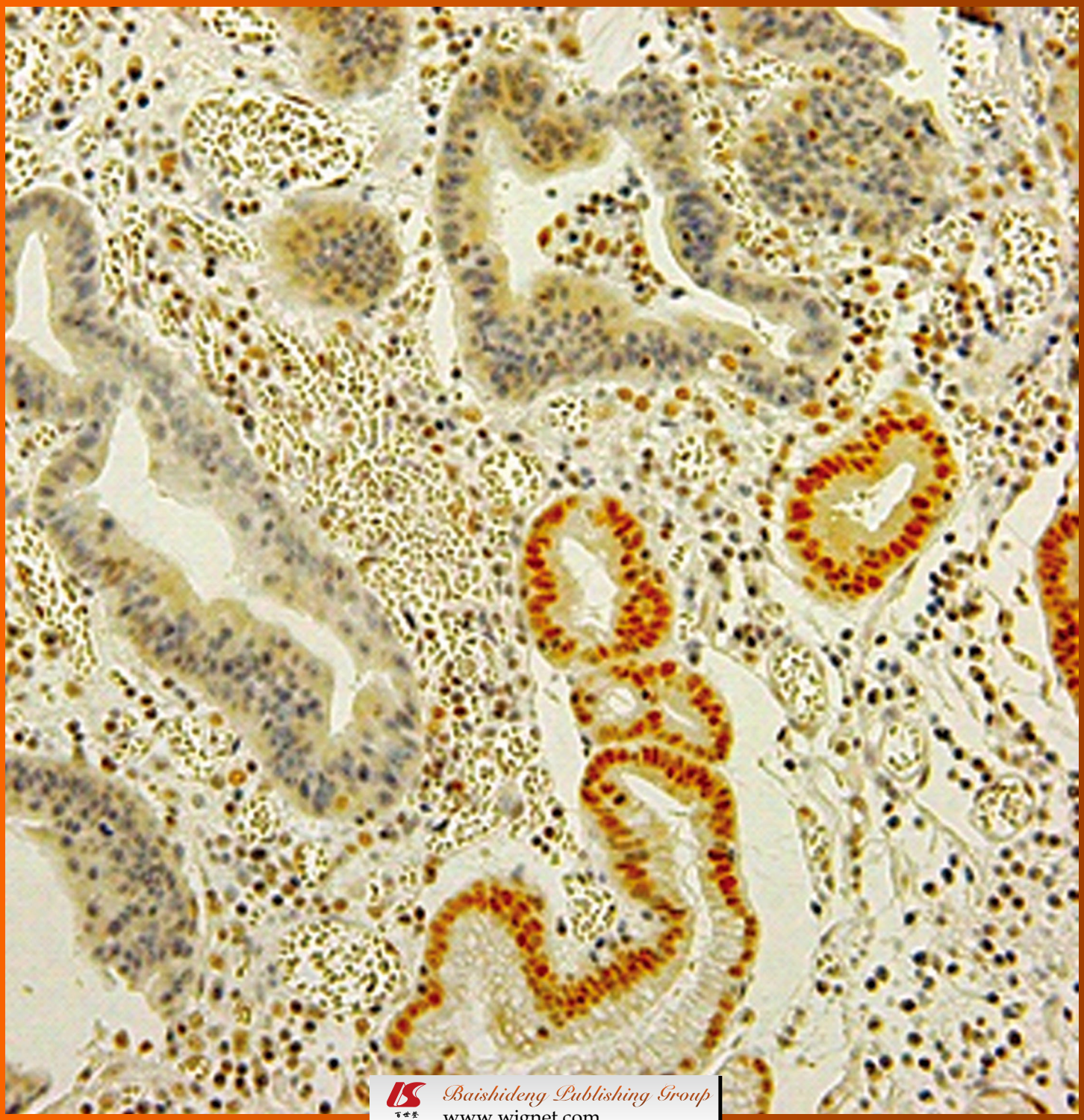

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EDITORIAL

Colorectal cancer screening in patients at moderately increased risk due to family history

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Abstract

Patients with a positive family history have an increased risk of colorectal cancer (CRC) and, in many countries, more intensive screening regimens, sometimes involving the use of colonoscopy as opposed to sigmoidoscopy or fecal occult blood testing, are recommended. This review discusses current screening guidelines in the United States and other countries, data on the magnitude of CRC risk in the presence of a family history and the efficacy of recommended screening programs, as well as ancillary issues such as compliance, cost-effectiveness and accuracy of family history ascertainment. We focus on the relatively common "sporadic" family histories of CRC, which typically imparts a mild to moderate elevation in the risk for CRC development in the proband. Defined familial syndromes associated with extremely high risks of CRC, such as hereditary non-polyposis colorectal syndrome or familial adenomatous polyposis, require specialized management approaches and are beyond the scope of this article. We will also not discuss colonoscopic surveillance in patients with a personal history of adenomas or CRC.

Key words: Colon cancer screening; Family history; Colonoscopy; Colon polyp

INTRODUCTION

Patients with a positive family history have an increased risk of colorectal cancer (CRC) and, in many countries, more intensive screening regimens, sometimes involving the use of colonoscopy as opposed to sigmoidoscopy or fecal occult blood testing, are recommended. This review discusses current screening guidelines in the United States and other countries, data on the magnitude of CRC risk in the presence of a family history and the efficacy of recommended screening programs, as well as ancillary issues such as compliance, cost-effectiveness and accuracy of family history ascertainment. We focus on the relatively common "sporadic" family histories of CRC, which typically imparts a mild to moderate elevation in the risk for CRC development in the proband. Defined familial syndromes associated with extremely high risks of CRC, such as hereditary non-polyposis colorectal syndrome or familial adenomatous polyposis, require specialized management approaches and are beyond the scope of this article. We will also not discuss colonoscopic surveillance in patients with a personal history of adenomas or CRC.

CURRENT GUIDELINES

In the United States, the earliest national guidelines were published in 1997 by the so-called Gastrointestinal Con-

sortium, a loose collaboration of gastroenterology and oncology groups[1]. These recommendations were updated subsequently by the three major gastroenterology societies, the American Gastroenterological Association (AGA)[2], the American College of Gastroenterology (ACG)[3] and the American Society of Gastrointestinal Endoscopy (ASGE)[4], each of whom published their most recent updates in 2008, 2009 and 2006 respectively. The AGA guidelines were published under the auspices of the Multi-Society Task Force on Colorectal Cancer (which also included representatives from the ACG and ASGE) in collaboration with the American Cancer Society and American College of Radiology. Other important groups, such as the US Preventive Services Task Force, have also offered guidelines applicable to individuals with a family history of CRC[5], but these lack operational detail, e.g., they do not specify when to start screening or how long the screening intervals should be.

In general, the guidelines from the US gastroenterology groups emphasize the use of colonoscopic screening, initiation of screening before age of 50 and shorter screening intervals for high-risk individuals with a significant family history of CRC (Table 1). However, there are some important differences between the guidelines. For persons with only a family history of non-advanced adenomas in first-degree relatives (FDRs) at any age or a family history of CRC in FDRs at age > 60 years, the ACG recommends only average-risk screening (starting at age 50 years), whereas the ASGE and AGA recommend initiating screening at age 40 years. In addition, while the ASGE relies heavily on colonoscopy as the preferred screening strategy in most patients with any family history, the AGA and ACG endorse the use of any acceptable screening modality (fecal occult blood testing, sigmoidoscopy or colonoscopy) in patients with less significant family histories. In the US, almost all public and private insurance plans cover CRC screening in patients with a family history of CRC, usually in the form of screening colonoscopy. With regard to Medicare, screening colonoscopy every 2 years is covered for so-called "high-risk" patients, a vaguely defined group that can include anybody with a first-degree or second-degree family history of CRC or "polyp".

From an international perspective, the World Gastroenterology Association presented comprehensive CRC screening guidelines in 2007[6]. These guidelines tailor the approach to each country, which is assigned to one of six "cascades" based on the epidemiology of CRC and economic resources available. For patients with a family history, screening colonoscopy every 5 years is recommended for countries in the upper socioeconomic tiers, while less expensive but still effective measures are recommended in countries with limited health care resources or endoscopic capacity. The Asian Pacific consensus guidelines published in 2008 also endorse early-onset screening in patients with a family history[7]. In addition, national guidelines are available for certain individual countries outside of the US, in particular Britain and Canada[8,9]. Germany and Poland already have large-scale screening colonoscopy programs, while many countries with national health insurance systems cover some CRC screening measures, most commonly fecal occult blood testing. In general, guidelines from other countries place less emphasis on the widespread use of screening colonoscopy and rely more on less expensive modalities, such as sigmoidoscopy or fecal occult blood testing[10].

EPIDEMIOLOGY

In the US, approximately 20% of CRC cases occur in patients with a first-degree family history of CRC. Because CRC is the third most common cancer in the US, 5%-10% of the general population have a first-degree family history of CRC[11,12] and almost 30% have a first- or second-degree relative (SDR) affected by CRC[12]. CRC is similarly prevalent in many other countries. Thus, recommendations on screening persons with a family history of CRC have widespread ramifications.

FAMILY HISTORY OF CRC

Based on mostly case-control or cross-sectional data, it is clear that a positive family history of CRC confers an increased risk for the development of CRC[13-21]. The few studies that did not show a significant increase risk were uncontrolled, small or of poor quality[22]. Most studies attribute the increased risk to earlier initiation of adenoma formation, but one study also showed that family history is associated with increased adenoma growth rates[23]. Large registry studies have confirmed that the risk of CRC in those with a family history is brought forward by about 10 years compared with those without a family history, implying that screening should start earlier in the former group[24]. However, there is some doubt as to whether or not screening recommendations should be different for those with relatives who developed CRC younger than 60 *vs* those whose relatives developed CRC at an older age. In one study, the former group did not demonstrate a higher incidence of advanced neoplasia on screening colonoscopy compared to the latter[25].

The increased risk associated with a family history of CRC has been investigated by several meta-analyses[26-28]. The earliest review included 27 studies and reported a relative risk of 2.25 if a patient has a FDR with CRC and 4.25 if there are multiple FDRs with CRC[28]. Another meta-analysis summarized data from 33 studies, showing that the elevated relative risk in the proband decreased as he or she aged, from 3.73 at age 40 years to 1.59 at age 70 years[26]. No difference was found between the impact of male and female affected relatives, nor between rectal *vs* colon cancer[26]. According to the most recently published meta-analysis, which summarized data from 59 studies, the absolute cumulative risk for CRC development between age 40-75 years is 4.7% for those with at least one affected SDR and 9.6% for those with at least one affected FDR[27]. It is suggested that the risk

Table 1 Colorectal cancer screening guidelines for patients with a family history[2-4]

Family history	ACG			ASGE			AGA		
	Screening initiation age (yr)	Screening modality	Screening intervals (yr)	Screening initiation age (yr)	Screening modality	Screening intervals (yr)	Screening initiation age (yr)	Screening modality	Screening intervals (yr)
2 FDRs with neoplasia[3]	40[1]	Colonoscopy	5	-	-	-	40[1]	Colonoscopy	5
1 FDR with CRC < 60[3]	40[1]	Colonoscopy	5	40[1]	Colonoscopy	3-5	40[1]	Colonoscopy	5
1 FDR with CRC ≥ 60[3]	50	Any	Average risk	40	Colonoscopy	10	40	Any	Average risk
1 FDR with adenoma < 60	50	Any	Average risk	40[1]	Colonoscopy	5	40[1]	Colonoscopy	5
1 FDR with adenoma ≥ 60	50	Any	Average risk	Not specified	Colonoscopy	10	40	Any	Average risk
2 SDRs with CRC[2]	-	-	-	50	Any	Average risk	40	Any	Average risk

[1]40 years old or 10 years younger than the age of diagnosis of the youngest affected relative, whichever is younger; [2]One second-degree relative (SDR) or third-degree relative in the case of the American Society of Gastrointestinal Endoscopy (ASGE) recommendations; [3]For the American College of Gastroenterology (ACG), either colorectal cancer (CRC) or advanced neoplasm (tubular adenoma ≥ 1 cm or any adenoma with villous or high-grade dysplastic features). The notation "1 first-degree relative (FDR) with CRC < 60" means "colorectal cancer in a first-degree relative with age of onset younger than 60 years". AGA: American Gastroenterological Association.

Table 2 Relative risk of colorectal cancer occurrence in a proband associated with different constellations of family history[33]

No. of FDRs with CRC	No. of SDRs with CRC	No. of TDRs with CRC	Relative risk (95% CI)
1	0	0	1.76 (1.63-1.89)
2	-	-	3.01 (2.66-3.38)
0	2	-	1.20 (1.05-1.38)
1	1	-	2.12 (1.90-2.35)
1	2	-	2.31 (1.80-2.93)
0	1	2	1.33 (1.13-1.55)

FDR: First-degree relative; SDR: Second- degree relative; TDR: Third-degree relative; CI: Confidence interval; CRC: Colorectal cancer.

conferred by a family history in siblings might be higher than the risk conferred by parents[27].

Many studies have reported that the risk of colorectal adenoma development is also increased in the presence of a family history of CRC[29-31], with a meta-analysis of 13 studies concluding that the overall relative risk was 1.7[32].

Many families have complex combinations of affected FDRs, SDRs and/or third-degree relatives (TDRs). A study using a large population database from Utah recently demonstrated that the risk changes with different constellation patterns of affected relatives (Table 2)[33]. In the presence of FDR family history, affected SDRs and TDRs can further increase risk to the proband. However, second- or three-degree family history alone increases the risk in the proband only slightly, to a clinically insignificant degree. The data also showed that risk is increased to 4.97 in those with both parents afflicted with CRC, and that older age of diagnosis (up to 70 years old) does not negate the increased risk in those with affected FDRs.

FAMILY HISTORY OF COLORECTAL ADENOMAS

Patients with a family history of colorectal adenomas also appear to exhibit increased risk[34,35], although some experts have expressed concerns that case-control studies reporting odds ratios purporting to reflect an increased risk of CRC in relatives of those with adenomas may actually be evaluating the reverse risk[3]. However, there is probably a true increase in risk, as evidenced by one prospective cohort study that showed an increased prevalence of large adenomas or CRC in FDRs of patients with large adenomas[36]. According to a meta-analysis, the relative risk of developing CRC in those with a family history of adenomas is 1.99[28]. The new ACG guidelines recommend only average-risk screening for patients with a family history of non-advanced adenomas. In contrast, the ASGE and AGA guidelines advise more aggressive screening regimens for patients with a family history of adenomas (of any size)[2,4]. Guidelines from countries outside the US generally do not recommend more aggressive screening for those with only a family history of adenomas[10].

EFFICACY OF SCREENING

In general, the yield of colonoscopy for detecting colorectal neoplasia is high in FDRs of patients with CRC[37-40], in many cases higher than that seen in matched patients without a family history[41-44]. However, there have been occasional studies reporting low yield[45], while some have disputed the usefulness of initiating screening at age 40 years[46].

The efficacy of screening colonoscopy at reducing CRC incidence and mortality specifically in patients with a family history has been well documented in non-randomized studies[47,48]. There have also been many large studies that included patients with and without a family history, showing improvement in CRC incidence and mortality with screening; however, none of these studies stratified results specifically for patients with a family history.

COMPLIANCE WITH SCREENING RECOMMENDATIONS

Surveys show that many primary care providers and gas-

troenterologists recommend screening colonoscopy starting at age 40 years for high-risk patients[49], while adherence to screening recommendations is variable in relatives of patients with CRC[50,51]. In general, African Americans with a family history are less likely to undergo appropriate screening than whites with a family history[52]. One study suggests that awareness of family history and increased risk can serve as a motivating factor for undergoing CRC screening[53]. In a recent study, we retrospectively reviewed the most recent 161 screening colonoscopies performed at our hospital involving patients with a family history of CRC in a FDR[54]. We found that 103 (64%) had not been referred for screening in compliance with guideline recommendations. Specifically, 92 (57%) had delayed initiation of screening (i.e., screening was started at an age much later than that recommended by the guidelines), 5 (3%) had premature initiation of screening, and 6 (4%) had screening with the wrong modality. Of cases involving delayed screening initiation, in 15 (16%) the patient was not under the care of a primary care provider at the time screening was supposed to have started, in 3 (3%) the patient refused screening despite recommendations by the primary care provider, and in 26 (28%) the patient was older than the recommended age by the time CRC was discovered in their relatives (usually siblings). The remaining patients had no discernible reason and it can surmised that many of these were not referred for screening appropriately because of knowledge defects in their primary care providers with regard to screening guidelines.

COST-EFFECTIVENESS

A decision analysis study showed that the cost-effectiveness of screening for the presence of a family history of CRC ranged from $18 000 to $51 000 per life-year gained[55]. There have been no cost-effectiveness studies that directly analyzed patients with a family history of CRC but because almost all cost-effectiveness studies have concluded that screening average-risk patients is cost-effective[56], it is likely that screening patients with a family history of CRC, in whom the prevalence of CRCs and neoplasia is higher, will be cost-effective.

ACCURACY OF FAMILY HISTORY REPORTING

For screening to be effective, the accuracy of any CRC family history must be assured. Several studies have looked at the reliability of patient self-reporting of family history, showing accuracy rates of 57%-83% for positive FDR history and 98%-99% for negative family history; as might be expected, accuracy for self-reporting of family history in SDRs or TDRs was lower (27%-67%)[57-60]. The accuracy of family history of colorectal adenomas is even more problematic. Subjects may not be aware of the size or histology of polyps found in relatives, thus making it difficult to derive accurate family histories of adenomas. For this reason, the ACG recommends that a family history of "polyps" should be treated as a family history of advanced neoplasia only if there is reasonable certainty that the polyp in the affected relative was indeed an advanced neoplasm, based on patient recall or medical records[3].

CONCLUSION

In conclusion, family history of CRC is a well established risk factor for CRC development in the proband and more aggressive screening regimens for such high-risk patients are well supported by available evidence and appear to be cost-effective. Compliance with current guidelines is still suboptimal and may be affected by under-reporting of positive family histories. These findings emphasize the importance of ongoing measures to improve screening compliance in high-risk patients.

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ORIGINAL ARTICLE

Expression of AID, P53, and Mlh1 proteins in endoscopically resected differentiated-type early gastric cancer

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Abstract

AIM: To analyze the expression of the tumor-related proteins in differentiated-type early gastric carcinoma (DEGC) samples.

METHODS: Tumor specimens were obtained from 102 patients (75 males and 27 females) who had received an endoscopic tumor resection at Tottori University Hospital between 2007 and 2009. Ninety-one cancer samples corresponded to noninvasive or intramucosal carcinoma according to the Vienna classification system, and 11 samples were submucosal invasive carcinomas. All of the EGCs were histologically differentiated carcinomas. All patients were classified as having *Helicobacter pylori* (*H. pylori*) infections by endoscopic atrophic changes or by testing seropositive for *H. pylori* IgG. All of the samples were histopathologically classified as either tubular or papillary adenocarcinoma according to their structure. The immunohistochemical staining was performed in a blinded manner with respect to the clinical information. Two independent observers evaluated protein expression. All data were statistically analyzed then.

RESULTS: The rates of aberrant activation-induced cytidine deaminase (AID) expression and P53 overexpression were both 34.3% in DEGCs. The expression of Mlh1 was lost in 18.6% of DEGCs. Aberrant AID expression was not significantly associated with P53 overexpression in DEGCs. However, AID expression was associated with the severity of mononuclear cell activity in the non-cancerous mucosa adjacent to the tumor (P = 0.064). The rate of P53 expression was significantly greater in flat or depressed tumors than in elevated tumors. The frequency of Mlh1 loss was significantly increased in distal tumors, elevated gross-type tumors, papillary histological-type tumors, and tumors with a severe degree of endoscopic atrophic gastritis (P < 0.05).

CONCLUSION: Aberrant AID expression, P53 overexpression, and the loss of Mlh1 were all associated with clinicopathological features and gastric mucosal alterations in DEGCs. The aberrant expression of AID protein may partly contribute to the induction of nuclear P53 expression.

Key words: Gastric cancer; Activation-induced cytidine deaminase; P53; Mlh1; Endoscopic resection

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INTRODUCTION

Gastric cancer (GC) is the second leading cause of cancer death and the fourth most common malignant tumor in the world[1]. The mortality rate associated with the disease is high, with a 5-year survival rate of approximately 20% being observed worldwide[2]. The 5-year survival rate for GC is over 50% in Japan[3]. One of the main factors limiting the survival rate is late tumor detection. Therefore, a better understanding of the clinicopathological characteristics in early GC (EGC) is critical. Infection with *Helicobacter pylori* (*H. pylori*), especially when "cag" pathogenicity island (cag PAI) positive, increases the risk of developing GC by more than 6-fold. Therefore, cag PAI is considered an important carcinogenic trigger[4]. Almost all *H. pylori* strains in Japan are cag PAI-positive[5]. Infection with *H. pylori* causes chronic inflammation of the gastric mucosa, which slowly progresses through the premalignant stages of atrophic gastritis, intestinal metaplasia and adenoma/dysplasia to GC[6]. The Japanese Research Society for Gastric Cancer has proposed that GC is divided into differentiated and undifferentiated types according to the degree of glandular formation by the tumor cells[7]. Additionally, each type of cancer might follow different genetic pathways during carcinogenesis[8]. The frequency of differentiated-type carcinomas among total EGC is approximately 60%. Therefore, differentiated-type early gastric carcinoma (DEGC) is considered to represent the initial phase of GC[9].

Gastric carcinoma results from the accumulation of genetic and epigenetic alterations[8]. The frequency of *MLH1* DNA methylation is 20%-30%[8,10] and the frequency of *P53* gene mutations is 25%-50%[8,11] in sporadic GC. *MLH1* is a DNA mismatch repair gene. Hypermethylation of the *MLH1* promoter region is the main cause of microsatellite instability (MSI) in primary GCs[12]. Activation-induced cytidine deaminase (AID) is a DNA- and RNA-editing enzyme that was originally identified as an inducer of somatic hypermutation and class-switch recombination in the immunoglobulin genes[13]. Previous reports indicate that AID transgenic mice develop malignant T-cell lymphomas and lung adenomas. This finding suggests that aberrant AID expression results in tumor-related gene mutations and might be a cause of human malignancy[14]. It has been reported that cag PAI-positive *H. pylori* infection causes the aberrant expression of AID in the gastric epithelium. Aberrant AID expression leads to the accumulation of nucleotide alterations in the *P53* gene[15]. Although the relationship between AID and Mlh1 is currently unclear, the expression of P53 has been reported to be inversely associated with Mlh1 loss in GC[8]. Elucidation of the relationship between the clinicopathological characteristics and the molecular events in EGC might improve the early detection, treatment, and surveillance of GC.

In this study, we evaluated AID, P53, and Mlh1 expression in endoscopically resected DEGCs and investigated their relationships with clinicopathological characteristics and background mucosa.

MATERIALS AND METHODS

Patient and tissue samples

Tumor specimens were obtained from 102 patients (75 males and 27 females) who had received an endoscopic tumor resection at Tottori University Hospital between 2007 and 2009 (Table 1). The mean age (± SD) was 70.6 ± 7.8 years (range: 55-92 years). The male patients were statistically younger than the female patients (69.4 ± 7.9 *vs* 74.2 ± 6.6, $P = 0.006$). We classified the DEGCs based on the Japanese classification of GC, 13th edition (7) according to location, macroscopic, and morphological types. The tumor location was defined as the upper third, middle third, or lower third of the tissue. The macroscopic type of DEGC was determined as elevated, depressed, or flat. All of the samples were histopathologically classified as either tubular or papillary adenocarcinoma according to their structure.

Ninety-one cancer samples corresponded to noninvasive or intramucosal carcinoma according to the Vienna classification system[16], and 11 samples were submucosal invasive carcinomas. All of the EGCs were histologically differentiated carcinomas. All patients were classified as having *H. pylori* infections by endoscopic atrophic changes or by testing seropositive for *H. pylori* IgG. Two experienced pathologists (Yashima K and Ito H) verified the pathological diagnoses. Moreover, we confirmed that these patients had no *H. pylori* eradication history. All specimens were assigned a new number without personal information to maintain anonymity. This study was approved by the institutional ethics committee of Tottori University (No. 314).

Evaluation of endoscopic gastric atrophy

All endoscopic examinations were performed using video scopes (model GIF-Q260; Olympus, Tokyo, Japan) and two endoscopists (Takeda Y and Yashima K) evaluated gastric atrophy according to the location of the atrophic border as described by Kimura *et al*[17]. A difference in the color and height of the gastric mucosa defines the border between the pyloric and fundic gland regions. We scored endoscopic gastric atrophy as marked (O2-O3), moderate (C3-O1) or mild (C1-C2). Previously, Takao *et al*[18] reported a significant correlation between endoscopic gastric atrophy (Kimura-Takemoto classification[17]) and the histological gastritis (updated Sydney system[19]). This suggests that the degree of endoscopic gastric atrophy can be considered as the grade of atrophic gastritis.

Evaluation of surrounding mucosal inflammation

We evaluated mononuclear cell activity in the non-can-

Table 1 Patients and tissue samples *n* (%)

	Total (*n* = 102)	Male (*n* = 75)	Female (*n* = 27)	Gender difference
Age (yr, mean ± SD)	70.6 ± 7.8	69.4 ± 7.9	74.2 ± 6.6	*P* = 0.006
Tumor size (cm)				
< 2.0	76 (74.5)	54 (72.0)	22 (81.5)	*P* = 0.332
⩾ 2.0	26 (25.5)	21 (28.0)	5 (18.5)	
Tumor location				
Upper third	23 (22.5)	19 (25.3)	4 (14.8)	*P* = 0.095
Middle third	40 (39.2)	32 (41.6)	8 (29.6)	
Lower third	39 (38.2)	24 (32.0)	15 (55.6)	
Gross tumor appearance				
Flat/depressed	63 (61.8)	47 (62.7)	16 (59.3)	*P* = 0.755
Elevated	39 (38.2)	28 (37.3)	11 (40.7)	
Histological type				
Tubular	88 (86.3)	66 (88.0)	22 (81.5)	*P* = 0.605
Papillary	14 (13.7)	9 (12.0)	5 (18.5)	
Depth of invasion				
Mucosa	91 (89.2)	66 (88.0)	25 (92.6)	*P* = 0.509
Submucosa	11 (10.8)	9 (12.0)	2 (7.4)	

cerous mucosa adjacent to a tumor and scored it as mild, moderate or marked according to the updated Sydney system[19].

Immunohistochemical staining

Paraffin-embedded sections (4 μm) were immunohistochemically stained with an anti-AID rat monoclonal antibody (EK2 5G9, Cell Signaling TECHNOLOGY, Danvers, CA, USA; dilution 1:400), an anti-P53 mouse monoclonal antibody (DO-7, Dakopatts, Copenhagen, Denmark; dilution 1:50), and an anti-Mlh1 mouse monoclonal antibody (G168-15, PharMingen, San Diego, CA, USA; dilution 1:50) using the avidin-biotin-peroxidase complex technique.

The immunohistochemical staining was performed in a blinded manner with respect to the clinical information. The sections were deparaffinized in xylene and rehydrated in ethanol. The sections were then immersed in a citrate buffer (0.01 mol/L, pH 6.0) and heated in a microwave oven for 20-30 min to retrieve antigens. The endogenous tissue peroxidase activity was blocked by incubation with 3% H_2O_2. The sections were subsequently incubated with primary antibody overnight at 4 ℃. As a negative control, the primary antibody was replaced with normal serum IgG at a similar dilution. The detection reaction followed the Vectastain Elite ABC kit protocol (Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine as the chromogen. The sections were counterstained with hematoxylin. The sections were incubated with biotinylated anti-rat or anti-mouse IgG and avidin-biotin-peroxidase. The sections were subsequently visualized using diaminobenzidine tetrahydrochloride. Two independent observers (Takeda Y and Yashima K) evaluated protein expression.

Assessment of AID immunostaining

The internal positive controls were lymphocytes of germinal centers in lymphoid follicles (Figure 1A). The follicles contain activated B cells and intensely stained positive for AID in all specimens. The cytoplasm was scored as positive when > 30% of tumor cells were stained as strongly as the germinal centers.

Assessment of P53 immunostaining

The tumors were scored as positive for P53 when a distinct nuclear immunoreaction occurred in > 25% of tumor cells[20] as shown in Figure 1B.

Assessment of Mlh1 immunostaining

The evaluation of Mlh1 expression was classified as being either normal or decreased (Figure 1C). Tissue specimens with definite nuclear staining in < 30% of the tumor cells were categorized as having decreased staining[21].

Statistical analysis

All data were statistically analyzed by the χ^2 test with Yates' correction, Fisher's test and the Mann-Whitney test (*U*-test) using Stat View 5.0 software (SAS Institute, Cary, NC, USA). Statistical significance was established at $P < 0.05$.

RESULTS

Frequency of aberrant AID, P53, and Mlh1 expression

Aberrant AID expression and P53 overexpression in DEGCs were detected in 35 (34.3%) cases. The loss of Mlh1 expression was observed in 19 (18.6%) cases. Among elderly patients (⩾ 65 years old), the loss of Mlh1 expression in DEGCs was significantly higher in female patients than in male patients [10/26 (38.5%) *vs* 6/54 (11.1%), *P* = 0.004] (Table 2).

Relationships between AID, P53 and Mlh1 expression

The overexpression of P53 was significantly more frequent in patients with Mlh1-positive tumors than Mlh1-negative tumors [33/83(39.7%) *vs* 2/19(10.5%), *P* = 0.015] (Table 3). The overexpression of P53 was not associated with aberrant AID expression (*P* = 0.657).

Table 2 Frequency of aberrant activation-induced cytidine deaminase, p53 and Mlh1 expression

	Total	Age < 65 yr (n = 22)			Age ⩾ 65 yr (n = 80)		
		Male	Female		Male	Female	
AID							
+	35	6	1	P = 0.689	16	12	P = 0.147
-	67	15	0		38	14	
P53							
+	35	11	0	P = 1.000	20	4	P = 0.086
-	67	10	1		34	22	
Mlh1							
+	83	18	1	P = 0.278	48	16	P = 0.004
-	19	3	0		6	10	-

AID: Activation-induced cytidine deaminase.

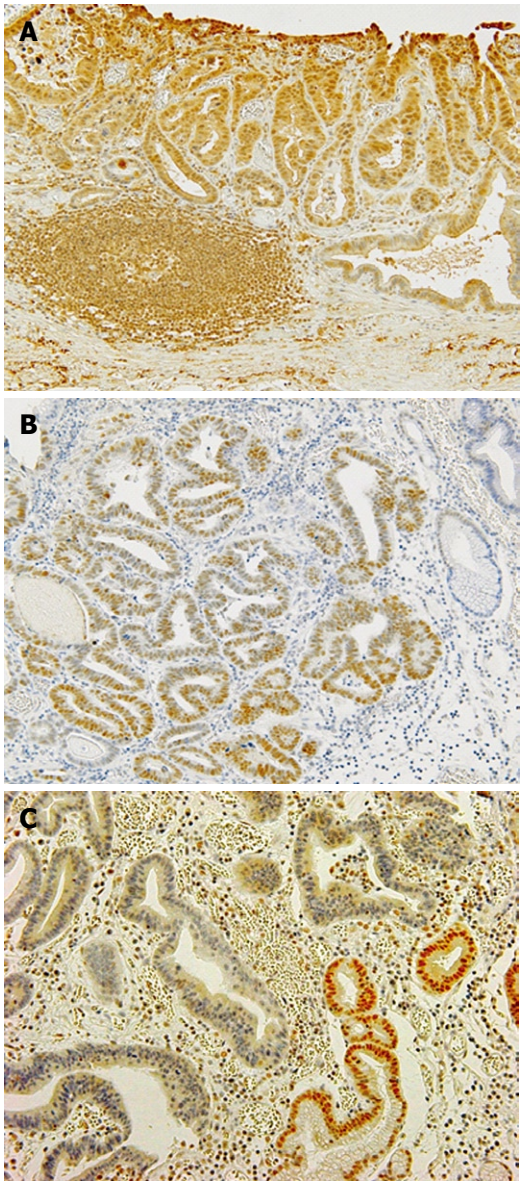

Figure 1 Representative findings of activation-induced cytidine deaminase, P53 and Mlh1 immunohistological stain in differentiated-type early gastric carcinoma. A: Positive activation-induced cytidine deaminase immunostaining in cytoplasm of differentiated-type early gastric carcinoma (DEGC); B: Overexpression of P53 in DEGC; C: No nuclear immunoreactivity for Mlh1 protein in DEGC.

Table 3 Relationships among activation-induced cytidine deaminase, P53 and Mlh1 expression

	AID			Mlh1		
	+	-		+	-	
P53						
+	11	24	P = 0.657	33	2	P = 0.015
-	24	43		50	17	

AID: Activation-induced cytidine deaminase.

Relationship of AID, P53, and Mlh1 expression with tumor features

The aberrant AID expression frequency was correlated with the location of DEGCs. However, there was no correlation between AID expression and tumor growth or histological type. The incidence of P53 overexpression in DEGCs was significantly more frequent in flat or depressed tumors than in elevated type tumors [28/64 (43.8%) *vs* 7/38 (18.4%), P = 0.009] (Table 4). The overexpression of P53 was found more often in tubular tumors than in papillary adenocarcinoma [34/88 (38.6%) *vs*. 1/14 (7.1%), P = 0.045]. A loss of Mlh1 expression was closely associated with distal location (P = 0.027), elevated gross type (P = 0.039) and papillary histological type (P = 0.033).

Relationships of AID, P53, and Mlh1 expression with background mucosa

Although aberrant AID expression was not related to gastric atrophy, mononuclear cell activity tended to be marked in the surrounding mucosa adjacent to DEGCs with aberrant AID expression (P = 0.064). The P53 expression in DEGCs was not associated with gastric atrophy and mononuclear cell activity in the surrounding mucosa. The loss of Mlh1 expression in DEGCs was associated with marked endoscopic gastric atrophy (P = 0.020) and mild mononuclear cell activity (P = 0.053) (Table 5).

DISCUSSION

The present study examined AID, P53 and Mlh1 expres-

Table 4 Relationships of activation-induced cytidine deaminase, P53 and Mlh1 expression with tumor features

	AID			P53			Mlh1		
	+	-		+	-		+	-	
Tumor location									
Upper third	4	19	$P = 0.067$	8	15	$P = 0.543$	22	1	$P = 0.027$
Middle third	13	27		16	24		34	6	
Lower third	18	21		11	28		27	12	
Tumor growth									
Flat/depressed	19	45	$P = 0.202$	28	36	$P = 0.009$	56	8	$P = 0.039$
Elevated	16	22		7	31		27	11	
Histological type									
Tubular	29	59	$P = 0.673$	34	54	$P = 0.045$	75	13	$P = 0.033$
Papillary	6	8		1	13		8	6	

AID: Activation-induced cytidine deaminase.

Table 5 Relationships of activation-induced cytidine deaminase, P53 and Mlh1 expression with background mucosa

	AID			P53			Mlh1		
	+	-		+	-		+	-	
Endoscopic gastric atrophy									
Mild	8	17	$P = 0.540$	9	16	$P = 0.376$	20	5	$P = 0.020$
Moderate	19	29		19	29		44	4	
Marked	8	21		7	22		19	10	
Mononuclear cell activity									
Mild	5	17	$P = 0.064$	4	17	$P = 0.232$	14	8	$P = 0.053$
Moderate	24	47		28	44		61	10	
Marked	6	3		3	6		8	1	

AID: Activation-induced cytidine deaminase.

sion in endoscopically resected DEGCs, and these results were compared with the clinicopathological characteristics and the surrounding mucosa. Aberrant AID expression in endoscopically resected DEGCs significantly correlated with marked mononuclear cell activity in tumor background mucosa but not with P53 overexpression. In addition, P53 expression significantly correlated with flat or depressed types of gross tumor appearance. The loss of Mlh1 expression correlated with elevated type, papillary type histology, distal location and severe endoscopic atrophic gastritis.

Infection with *H. pylori* triggers aberrant AID expression in the gastric epithelium, which leads to the accumulation of altered nucleotides in the *P53* gene[15,22,23]. The rate of aberrant AID expression in DEGC (34.3%) was slightly higher than the 26.9% and 22.5% described in two previous reports[23,24]. The variability in the findings may be caused by differences in the stage of carcinoma progression and the degree of tumor differentiation. All of our data were obtained from endoscopically resected, well-differentiated early carcinomas.

Previously, Kim *et al*[23] found a significant association between aberrant AID expression and the nuclear overexpression of P53 in various types of GCs. However, we did not find a relationship between aberrant AID expression and P53 overexpression in DEGCs. Similarly, Goto *et al*[24] found no correlation between AID and P53 in early differentiated and poorly differentiated GCs. There are several possible explanations for these different findings. One explanation is that nonsense mutations were considered to be false-negative. Additionally, P53 protein could accumulate to repair damaged DNA in false-positive cells without *P53* mutations[25]. The rate of P53 expression might also increase with tumor progression[26]. Moreover, P53 protein might become altered through cigarette smoking, as in lung and esophageal carcinogenesis[27,28]. Further investigation is needed to clarify the correlation between the expression of P53 and aberrant AID expression.

The expression of AID in gastric epithelial cells could be altered by the direct action of *H. pylori* macromolecules through the type IV secretion system encoded by cag PAI[29]. Additionally, *H. pylori* infection is associated with inflammatory cytokines, such as tumor necrosis factor α, that are produced during gastric inflammation[15]. Furthermore, AID expression in tumors such as hepatocellular carcinoma, cholangiocarcinoma and colon cancer is also mediated by proinflammatory cytokine stimulation *via* nuclear factor κB[30-32]. Aberrant AID expression correlates with chronic active inflammation, glandular atrophy and intestinal metaplasia in the non-neoplastic gastric mucosa[24]. The present study found that aberrant AID expression in tumors correlated with mononuclear cell activity in the mucosa surrounding the tumor, which would support the mechanisms of AID expression.

The 33.7% frequency of P53 overexpression in the

DEGC was consistent with previous findings[8]. The expression of P53 was associated with flat or depressed macroscopic tumor features but not with the other clinicopathological features of age, gender, or location and tumor size. In agreement with our results, Sasaki *et al*[9] also demonstrated that P53 overexpression is more frequent in depressed-type differentiated GCs.

Several epigenetic alterations in GC have been described[10,16]. DNA methylation of *MLH1* promoter region CpG islands is closely associated with a loss of Mlh1 expression in GCs that exhibit MSI[16]. *MLH1* hypermethylation is evident in 20%-28% of differentiated carcinomas[10,33]. The reported frequency of negative Mlh1 expression in both early and sporadic GC ranges from 13%-20%[34,35]. In the present study, the frequency of lost Mlh1 expression in DEGCs was 18.6%. Our study and previous studies have shown that GCs with reduced Mlh1 expression are statistically more prevalent among elderly women. Previous reports have suggested that[36], high-frequency MSI (MSI-H) GCs are characterized by an antral location and proliferation. A loss of Mlh1 expression was associated with the lower third of the stomach and elevated gross type in our study. Additionally, our findings were consistent with the Guos report[37], which showed a higher prevalence of MSI-H in papillary type GC than in early well-differentiated carcinoma.

Chronic gastritis induced by *H. pylori* infection usually progresses to atrophic gastritis, which is an established risk factor for GC. The risk increases with the degree and the extent of atrophic gastritis. However, no clinicopathological studies regarding the relationship between molecular events and the degree of endoscopic atrophy in patients with GC have been published. Factors such as aging, dietary habits, alcohol consumption, cigarette smoking and autoimmunity promote atrophic gastritis[38,39]. The frequency of Mlh1 loss increases in tumors that cause a severe degree of endoscopic atrophic gastritis. Our results suggest that several factors are involved in the gastric atrophic changes found in patients with DEGC accompanied by aberrant Mlh1 expression. However, more studies are required to identify the mechanism of this association. Moreover, significantly less mononuclear cell infiltration was evident in patients with DEGC that had lost Mlh1. These results might be a consequence of a reduction in *H. pylori* density accompanied with severe glandular atrophy, which might contribute to reduced inflammatory infiltration.

In conclusion, we investigated the relationships between AID, P53 and Mlh1 expression, clinicopathological characteristics, and mucosal alterations. Our results suggest that aberrant AID expression may partly contributes to P53 overexpression.

COMMENTS

Background

Helicobacter pylori infection causes aberrant activation-induced cytidine deaminase (AID) expression in gastric epithelial tissues, which results in alterations in various tumor-related genes. However, the precise molecular mechanisms underlying gastric carcinogenesis are not fully understood, particularly in endoscopically resected early gastric tumors.

Research frontiers

In this study, the authors analyzed the expression of the tumor-related proteins: AID, P53, and Mlh1 in 102 differentiated-type early gastric carcinoma (DEGC) samples obtained by endoscopic resection.

Innovations and breakthroughs

The authors found that the rates of aberrant AID expression and P53 overexpression were both 34.3% in DEGCs. The expression of Mlh1 was lost in 18.6% of DEGCs. Aberrant AID expression was not significantly associated with P53 overexpression in DEGCs. However, AID expression was associated with the severity of mononuclear cell activity in the non-cancerous mucosa adjacent to the tumor. The rate of P53 expression was significantly greater in flat or depressed tumors than in elevated tumors. The frequency of Mlh1 loss was significantly increased in distal tumors, elevated gross type tumors, papillary histological type tumors, and tumors with a severe degree of endoscopic atrophic gastritis.

Applications

Collectively, the data of this study suggested that aberrant AID expression, P53 overexpression, and the loss of Mlh1 were all associated with clinicopathological features and gastric mucosal alterations in DEGCs. Furthermore, the aberrant expression of AID protein may partly contribute to the induction of nuclear P53 expression.

Peer review

Takeda *et al* analyzed expression of the tumor-related proteins: AID, P53, and Mlh1 in 102 DEGC samples obtained by endoscopic resection and found aberrant AID expression, P53 overexpression and the loss of Mlh1 expression were associated with some of the clinicopathological features and gastric mucosal alterations in DEGCs, and that the aberrant expression of AID protein might partly contribute to the induction of nuclear P53 expression.

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BRIEF ARTICLE

DNA methylation patterns in alcoholics and family controls

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Abstract

AIM: To assess whether DNA methylation patterns in chronic alcoholics are different from non-alcoholic sibling controls.

METHODS: We examined the methylation patterns in DNA samples from 25 chronic alcoholics and 22 matched siblings as controls (one per family). DNA was extracted from peripheral blood and analyzed for differences in the methylation patterns after bisulfite-conversion. We used the Illumina GoldenGate Methylation Cancer Panel I (Illumina, San Diego, CA), which probes the methylation profile at 1505 CpG sites from 807 cancer related genes. We excluded the 84 X-chromosome CpG sites and 134 autosomal CpG sites that failed to show a within sample reliability score of at least 95% for all samples, leaving 1287 autosomal CpG sites (associated with 743 autosomal genes) with reliable signals for all samples. A methylation score was calculated as the average methylation for the 1287 CpG sites examined. Differences were assessed by a two-sample t-test. We also examined the average sib pair differences in methylation scores at each of the 1287 sites. All analyses were performed using SPSS, version 9.0, $P < 0.05$ was considered significant.

RESULTS: Methylation levels at the 1287 CpG sites averaged 28.2% for both alcoholics and controls. The mean difference in methylation scores between alcoholic and non-alcoholic sibs by CpG site was < 1% with small inter-individual variances; and only 5 CpG sites had an average sib difference > 5%. Subgroup analysis showed that methylation scores were significantly lower for the alcoholic-dependent subjects who smoked compared to their non-smoking unaffected siblings. Specifically, among smokers who are alcoholic, global methylation indices were significantly lower than in non-alcoholic sib controls, whereas among non-smoking alcoholics, the global indices were significantly higher ($P = 0.008$).

CONCLUSION: Although we observed no effect of alcoholism alone on DNA methylation, there is a decrease in alcoholics who smoke, suggesting a mechanism for alcohol-tobacco synergy for carcinogenesis.

Key words: DNA methylation; Alcohol; Epigenetics; Cancer; Carcinogenesis; Smoking; Cigarettes; Tobacco

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INTRODUCTION

Epigenetics is the study of heritable differences related to changes in gene expression that are not due to differences in DNA sequences themselves. Although still in its infancy, epigenetics is expanding rapidly as a field of study. DNA methylation, one of the two main types of epigenetic inheritance, is involved in many physiological and pathophysiological conditions, including regulation of gene expression and silencing of repeat elements in the genome. Epigenetic mechanisms have been implicated in the long term memory formation by neurons and are a growing area of research in diseases such as Alzheimer's dementia[1]. DNA methylation is thought to play important roles in many diseases, including multiple sclerosis, diabetes mellitus, schizophrenia, alcohol dependence and cancer[2-6].

It has been shown that global methylation status in peripheral blood monocytes is associated with plasma homocysteine levels in healthy individuals. The importance of homocysteine to DNA methylation status stems from the fact that homocysteine is a precursor of S-adenosyl methionine, which acts as the methyl donor when cytosine residues in the dinucleotide sequence CpG are methylated by DNA methyltransferases. Chronic alcoholics commonly have elevated homocysteine levels. Bönsch *et al*[7], showed associations among alcohol-associated elevated plasma homocysteine levels, global methylation levels assayed by difference in CpG methylation sensitive vs. insensitive restriction enzyme (Hpall/Mspl) digestion, and the subsequent expression of DNMT mRNAs in alcoholic patients, compared to controls. These findings support the hypothesis that ethanol exposure increases global levels of DNA methylation and suggests that changes in DNA methylation may result in changes in gene expression. Support for this hypothesis includes several reports of DNA hypermethylation associated with alcohol use at specific individual genes in peripheral blood cells[8-10]. Other studies have identified changes in methylation associated with smoking, suggesting both alcohol and smoking may contribute to changes in DNA methylation[11,12]. In all likelihood, many more genes whose levels of expression are partially controlled by the methylation status of the DNA in their promoters are yet to be discovered.

Changes in DNA methylation are recognized as one of the most common forms of molecular alteration in human neoplasia[13,14]. Hypermethylation of CpG islands located in the promoter regions of tumor suppressor genes has been firmly established as a mechanism for gene inactivation in cancers[15,16]. In contrast, global hypomethylation of genomic DNA[17] and loss of IGF2 imprinting were observed in tumor cells[18] and a correlation between hypomethylation and increased gene expression was reported for many oncogenes[19,20]. In addition, monitoring global changes in DNA methylation has been used for molecular classification of cancers[21,22]. Gene hypermethylation has been correlated with clinical risk groups for neuroblastoma[23], as well as with hormone receptor status and response to tamoxifen in breast cancer[24,25]. Therefore, it may be feasible to use methylation markers to classify and predict cancer risk, different kinds or stages of cancer, cancer therapeutic outcomes and patient survival.

Alcoholism and cancer risk

About 3.6% of all cases of cancer and a similar proportion of cancer deaths are attributable to heavy consumption of alcohol. These figures are higher in selected regions of the world, in particular in Central and Eastern Europe. Among women, 60% of cancers attributable to alcohol use occur in the breast[26]. Chronic excessive alcohol consumption is the strongest risk factor for upper aerodigestive tract (UADT) cancer (oral cavity, pharynx, hypopharynx, larynx and esophagus)[27]. Chronic and heavy alcohol use also increases the risk for cancer of the liver, colon, rectum and breast[28]. Many epidemiological studies have demonstrated a correlation between chronic and heavy alcohol ingestion and the occurrence of cancer in these organs[29-31]. Because the ingestion of all types of alcoholic beverages is associated with an increased cancer risk, more likely than not, ethanol itself is the crucial compound that increases cancer risk, rather than congeners (propanol, butanol, pentanol) or other additives. The exact mechanisms of ethanol-associated carcinogenesis have remained obscure.

Multiple mechanisms are believed to be involved in alcohol-associated cancer development of the UADT, including the effect of acetaldehyde (AcH the first metabolite of ethanol oxidation), induction of cytochrome P-4502E1 leading to the generation of reactive oxygen species, and enhanced procarcinogen activation, modulation of cellular regeneration, and nutritional deficiencies. Folate deficiency, primarily the consequence of low dietary intake and destruction by AcH, is common in alcoholics and contributes to the inhibition of transmethylation, which is an important factor in the regulation of genes involved in carcinogenesis. Acetaldehyde also decreases DNA repair mechanisms and the methylation of cytosine in DNA. However, it has been shown recently that chronic alcoholics have significantly increased levels of genomic DNA methylation in peripheral blood mononuclear cells (PBMC), compared to samples from unrelated volunteer blood donors[7].

Most studies to date have examined changes in global methylation in alcohol users or methylation changes at a few candidate genes, rather than at a broader panel of specific sites. This study was designed specifically to obtain preliminary data on the methylation status in PBMC of genes known or suspected of playing a role in cancer development. The primary aim was to assess the change in global DNA methylation levels at these gene specific sites in well-characterized chronic alcoholics and to compare it to suitably matched non-alcoholic family members as controls. We also wanted to explore whether there are observable, meaningful differences in methylation patterns between the two groups at different gene loci and whether there are relationships between life time alcohol use and the degree or pattern of DNA methylation.

MATERIALS AND METHODS

We examined the methylation patterns in DNA samples from 25 chronic alcoholics and 22 of their non-alcoholic biological siblings. We utilized the resources available through the UCONN Alcohol Research Center of UCHC to help us identify suitable alcohol-dependent subjects and their non-alcohol-dependent family members to serve as controls. The kindreds studied have been well characterized and followed longitudinally. They are enrolled in the long-standing Collaborative Study on the Genetics of Alcoholism[32,33]. After IRB approval, suitable subjects were identified and informed consent for participation in this study was obtained.

The alcohol-dependent subjects were at least 21 years of age and had a history of alcohol use for at least 5 years. All subjects were interviewed using the Semi-Structured Assessment for the Genetics of Alcoholism, a reliable and valid psychiatric diagnostic instrument[34]. Alcohol-dependent subjects met the DSM-IV diagnosis of alcoholic dependence. Males were consuming at least 15 drinks per week or 5 or more standard drinks in a day and females at least 8 or more drinks per week or 4 or more standard drinks in a day within the past year. Non-alcohol-dependent biological siblings of the subjects served as controls. The controls were screened for heavy alcohol use or history of cancer by self-reported questionnaires. They were required to have had a normal physical examination and no personal history of any kind of cancer other than superficial skin cancer. We excluded any subjects with known genetic abnormalities or chronic liver diseases (other than alcohol-related liver disease) and subjects with known nutritional disorders and/or anemia, which may have served as confounding variables. The sample examined included 22 sibships comprised of 25 probands and 22 siblings (3 sibships included 2 probands).

DNA methylation analysis

DNA was prepared from peripheral blood samples using a commercial kit (Gentra PureGene, Qiagen, Valencia, CA) and 500 ng of each DNA sample was bisulfite reacted using the EZ-96 DNA methylation-gold kit from Zymo Research (Orange, CA).

We used a high-throughput single nucleotide polymorphism genotyping system[35] for DNA methylation detection, based on genotyping of bisulfite-converted genomic DNA. This technology, developed by Illumina, combines a miniaturized bead-based array platform, a high level of assay multiplexing, and scalable automation for sample handling and data processing. We used the Illumina GoldenGate Methylation Cancer Panel I (Illumina, San Diego, CA), which probes the methylation profile at 1505 CpG sites from 807 genes selected by the manufacturer, based on their relevance to carcinogenesis. This assay is reported to have a sample replicate variation of $< 6\%$[36] and can resolve a 10% or greater methylation difference with 95% confidence.

We excluded the 84 X-chromosome CpG sites in the Illumina Cancer Panel because the methylation levels for X-chromosome sites vary greatly by sex [the X-chromosome (Lyon) inactivation in females is associated with methylation of CpG-rich islands[37]]. We also excluded from analysis 134 autosomal CpG sites that did not give an assay reliability score of at least 95% for all samples, leaving 1287 autosomal CpG sites with reliable signal for all samples. The included 1287 CpG sites were associated with 743 autosomal genes.

Statistical analysis

For each participant, we calculated a methylation score by computing the average methylation over the 1287 CpG sites examined. Differences in the mean methylation scores between the two samples were assessed by a two-sample *t*-test. We also examined the average sib pair differences in methylation scores at each of the 1287 sites evaluated with use of a paired *t* test. All analyses were performed using SPSS, version 9.0, $P < 0.05$ was considered a statistically significant result.

RESULTS

A total of 25 alcoholics and 22 matched controls (one control per family) were recruited for this study. The average age of probands and controls was not significantly different. Probands were more likely to be male (Fisher's exact test, $P = 0.004$). Three sib pairs contained 2 probands. As anticipated, the alcohol-dependent subjects had significantly higher amounts of alcohol use, both in terms of days (frequency) and drinks (quantity) per week (Table 1). Bisulfite reacted DNA was examined at 1421 autosomal CpG sites contained on the Illumina DNA methylation chip. Analysis was limited to the 1287 probes which generated valid test signals (95% quality confidence signal) from all samples. Methylation levels at the 1287 CpG sites averaged 28.2% for all samples combined. The mean methylation score was not significantly different between the alcohol-dependent subjects and their unaffected siblings (Table 2). The mean difference in methylation scores between affected and unaffected sibs by CpG site

Table 1 Selected demographic and alcohol-use features at baseline

	Alcohol-dependent siblings (n = 25)	Non-alcoholic siblings (n = 22)
Age (yr) (SD, range)	40.7 (9.5, 24-54)	40.6 (11.4, 21-59)
Gender (M:F)	18:7	4:18
Current smoker	14	7
Race: EA, AA, NA	14, 10, 1	13, 8, 1
Hispanic Ethnicity	2	3
Past 12-mo drinking (mean ± SD)		
Drinking days per week	3.91 ± 1.95[b]	0.95 ± 1.18
Drinks per drinking day	7.90 ± 4.01[b]	2.68 ± 1.34
Drinks per week	34.0 ± 30.8[b]	2.7 ± 3.6
[1]Heavy drinking days per week	2.53 ± 2.66[b]	0.18 ± 0.34

[1]"Heavy drinking days" were defined as days in which men consumed more than 10 drinks and women more than 8 drinks. [b]*t*-test $P < 0.001$ *vs* non-alcoholic siblings. EA: European American; AA: African American; NA: Native American.

Table 2 Global methylation scores

	Alcohol-dependent siblings	Non-alcoholic siblings
Global methylation index for 1287 CpG sites		
Mean methylation (SD)	0.282 (0.016)	0.282 (0.012)
Median methylation (SD)	0.082 (0.010)	0.079 (0.009)
Range	0.01-0.97	0.01-0.97
Sib pair difference in global methylation level at each of 1287 sites (alcoholic minus non-alcoholic sibling methylation level)		
Mean difference (SD)	0.00005 (0.019)	
Replicate pair difference in methylation level at each of 1287 sites (3 non-alcoholic and alcoholic siblings with replicate bisulfite treatment and methylation quantification)		
Mean difference (SD)	0.0008 (0.006)	0.001 (0.003)

No global measures of methylation significantly differ between groups.

was < 1% (Table 2) with a tight distribution, and only 5 CpG sites had an average sib difference > 5% (Figure 1). The sib difference and t-test statistic for these 5 CpG sites are listed in Table 3. Finally, as a test of the assay's reproducibility, we performed replicate bisulfite conversion and methylation assays for DNA samples from alcoholics and non-alcoholic participants from 3 sibships. The mean difference in replicate sample methylation for the 1287 CpG sites was less than 1% (Table 2).

Because tobacco use may also affect methylation levels, we conducted a subgroup analysis comparing the global methylation sib pair differences for sib pairs in which neither smoked (n = 7), those in which both smoked (n = 7), and sib pairs for which the proband smoked and the control sib did not (n = 7) (in two sib pairs, the control sib but not the alcoholic sib smoked; smoking status was not available for one proband). We found that, for the two groups of sib pairs concordant for smoking status, compared with the non-concordant group, the alcohol-dependent subjects had higher average methylation levels at the 1287 sites examined (F = 284, df = 2, $P < 0.001$). Similarly, for non-smoking sib pairs, in 6 of 7 pairs, alcoholic subjects had a higher average methylation index. In contrast, for discordant pairs with an alcoholic smoker, in 6 of 7, the alcoholic subject had a lower average methylation index than the non-alcoholic, non-smoking sibling (χ^2 = 8.2, df = 2, P = 0.017) (Table 4).

Table 3 Alcoholic minus non-alcoholic sib differences in methylation scores at 5 CpG sites with average difference in methylation frequency > 0.05

Gene symbol	Illumina CpG probe ID	Average Sib difference	Paired t-test statistic (2-tailed)	P value
LTA	820	0.083	2.46	0.021
CRK	3392	0.068	3.18	0.004
GSTM1	4902	0.054	1.82	0.081
HPN	4931	-0.084	-2.14	0.043
MSH3	2787	-0.052	-1.35	0.189

LTA: Lymphotoxin α precursor; CRK: v-crk sarcoma virus CT10 oncogene homolog isoform b; GSTM1: Glutathione S-transferase M1 isoform 1; HPN: Hepsin (transmembrane protease, serine 1); MSH3: MutS homolog 3.

DISCUSSION

The major findings of this study are two-fold: (1) Contrary to our *a priori* major hypothesis, there was no difference in average CpG methylation scores between alcohol-dependent subjects and non-alcoholic siblings; and (2) However, in a secondary analysis, we did find a small but significant decrease in PBMC methylation scores in the alcoholic subjects who smoked, when compared to their non-alcohol dependent siblings who did not smoke (Table 4). Thus, despite heavy, chronic and ongoing alcohol use in the alcohol-dependent probands, we found no effect on average methylation of the DNA of PBMCs for a set of 1287 CpG sites associated with 743 genes implicated in carcinogenesis. This is in contrast to results reported by Bönsch *et al*[7] who have shown a global CpG DNA hypermethylation in chronic alcoholics. However, in previous work, results among alcoholics were compared to a random, unrelated non-alcoholic control population and genes particularly relevant to cancer development were not studied. Gender and race have recently been reported to influence global genomic methylation in peripheral blood[38], emphasizing the importance of carefully matched controls in studies of this type. We believe that our family controls are a unique strength of our results.

Others have shown that global leukocyte DNA hypomethylation is associated with the risk of developing breast cancer[39]. In a mouse model of cutaneous carcinogenesis, it has been shown that the degree of DNA hypomethylation of genomic DNA increases as lesions progress from a benign to invasive cancers[40]. The discordant results can be explained by the fact that hypomethylation is most relevant when it occurs in the coding regions

Table 4 Global methylation score sib-pair differences for non-smokers *vs* sibship with alcoholic tobacco user (7 sib pairs)

	Sib pair concordance for smoking status		
	Concordant		Discordant
	Both non-smokers	Both smokers	Proband smokes
Mean (SD) sib pair difference in methylation at each site (alcoholic minus non-alcoholic sibling methylation level)	+0.006 (0.018)	+0.010 (0.020)	-0.009 (0.025)

Mean sib pair difference for 1287 markers, ANOVA: F = 284 (df = 2), $P < 0.0001$. Among concordant non-smoking sib pairs, for 6 of 7 pairs alcoholic subject had higher methylation index among concordant. Five of 7 smoking sib pairs alcoholic subjects had higher methylation index. Among discordant pairs with an alcoholic smoker, 6 of 7 alcoholic subjects had a lower methylation index than non-alcoholic siblings.

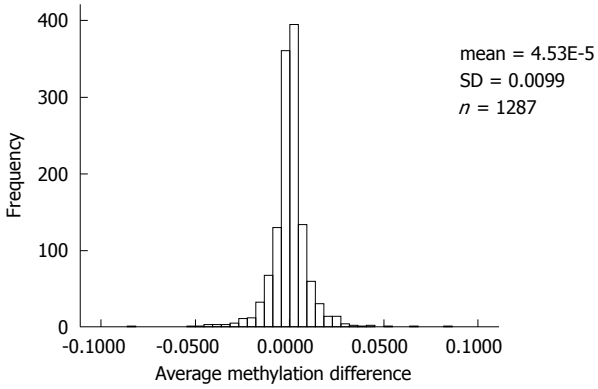

Figure 1 Frequency histogram of average within sib pair difference in methylation at 1287 CpG sites methylation level of alcoholic minus non-alcoholic sibling.

of the genes. In contrast to prior global CpG methylation analysis with respect to heavy and chronic alcohol use, our study found no meaningful change in levels of methylation at specific CpG sites of potential relevance to cancer-related genes, when results were compared to those of non-alcoholic siblings.

The combination of alcohol and tobacco use is known to be synergistic in markedly increasing the risk of development of malignancies of the UADT, especially squamous cell carcinomas of esophagus, lung and oropharynx[41-44]. Our finding of increased CpG methylation among alcoholics vs. non-alcoholic siblings for those 14 sib pairs concordant for smoking status, corrected for the status of their sibs (Table 4), is thus of much interest. If confirmed in larger number of subjects and in several other samples, it will suggest that factors other than hypomethylation of DNA accounts for the well established synergism of alcohol and tobacco in the pathogenesis of cancer of UADT.

Our study had several limitations. Perhaps most important is the small sample size, which, due to limitations in time and funding, was only about half as large as we had hoped. Secondly, this is not a genome-wide study, but rather examines only a select group of candidate genes, albeit genes pre-selected for their known relevance to cancer development. Nonetheless, the genes examined may not be as important in early stage carcinogenesis and/or may be affected by other epigenetic factors such as histone modifications. Another unavoidable limitation was that most alcoholics were men, whereas most non-alcoholic siblings were women. Thus, although matched genetically by family, alcoholic subjects and controls were not closely matched by gender.

A major strength of this study is the inclusion of biological siblings unaffected by alcoholism as controls. Also, the tumor genes included on the Illumina Cancer Methylation Assay chip have been well characterized previously as related to cancers of the UADT. We excluded from analysis the CpG sites related to the X and Y chromosomes that could have had a confounding effect on our results. This is supported by a recent study by Zhang *et al*[38] showing significantly lower global genomic DNA methylation in females. It is thought that X chromosome inactivation in women may diminish the capacity for methylating autosomal loci[45].

In summary, our study did not reveal any significant differences in the average methylation score between alcoholic and non-alcoholic siblings associated with 743 genes implicated in carcinogenesis. However, subgroup analysis did show a significantly decreased methylation of genes important in cancer development among alcoholics who smoked, compared to their non-alcoholic siblings who did not smoke. This finding needs confirmation in larger independent samples. It would also be prudent to consider *a priori* the combined effect of alcohol and smoking when planning future studies examining the effects of alcohol on DNA methylation.

COMMENTS

Background

DNA methylation is thought to play an important role in cancer development. Chronic and heavy alcohol has long been associated with a variety of cancers and has recently been associated with increased DNA methylation levels.

Research frontiers

The authors planned this study to assess whether DNA methylation patterns in chronic alcoholics are different from non-alcoholic siblings who served as controls for comparison.

Innovations and breakthroughs

The major findings of this study are two-fold: (1) Contrary to our *belief*, there was no difference in average CpG methylation scores between alcohol-dependent subjects and non-alcoholic siblings; and (2) However, in a secondary analysis, we did find a small but significant decrease in methylation scores of DNA from peripheral blood mononuclear cells in the alcoholic subjects who smoked, when compared to their non-alcohol dependent siblings who did not smoke. Thus, despite heavy, chronic and ongoing alcohol use in the alcohol-dependent subjects, we found no effect on average methylation for the set of

743 genes examined, which have previously been implicated in carcinogenesis. This is in contrast to results reported by Bönsch *et al* who reported a global DNA hypermethylation in chronic alcoholics, albeit not adjusted for results from controls from the same families.

Applications

Subgroup analysis did show significantly decreased methylation of genes important in cancer development among alcoholics who smoked, compared to their non-alcoholic siblings who did not smoke. This finding needs confirmation in larger independent samples. It would also be prudent to consider a priori the combined effect of alcohol and smoking when planning future studies examining the effects of alcohol on DNA methylation.

Terminology

DNA Methylation: It refers to the addition of a methyl group to the DNA at specific locations, namely, the cytosine residues of CpG dimers. DNA methylation is thought to regulate a number of cellular processes in the human body and also to influence the development of cancer when it occurs at specific sites.

Peer review

The study was well planned and conducted. The conclusions drawn are supported by the results. The study however is limited by its limited sample size and the fact that it examines only a select group of genes that have been associated to cancer development. A major strength of this study is the use of siblings as controls to adjust for any differences in the DNA methylation status that may be due to inherent genetic factors that differ among different kindreds.

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BRIEF ARTICLE

Serum M2-pyruvate kinase: A promising non-invasive biomarker for colorectal cancer mass screening

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Abstract

AIM: To explore the value of serum M2-pyruvate kinase (M2-PK) in colorectal cancer (CRC) mass screening.

METHODS: We conducted a molecular epidemiology study in Hangzhou, China, from year 2006 to year 2008. Serum samples were collected from 93 CRC, 41 advanced adenomas, 137 adenomas, 47 non-adenomatous polyps, and 158 normal participants in a community setting. Serum M2-PK and carcinoembryonic antigen (CEA) were measured using Enzyme-linked immunosorbent assay. SPSS 16.0 software was used to perform data analysis. Area under the receiver operating characteristic curve (AUC), sensitivity, and specificities were estimated for serum M2-PK in diagnosis of colorectal lesions and compared with CEA.

RESULTS: Average serum M2-PK value among 158 normal people was 2.96 U/mL and not affected by gender (P = 0.47) or age (P = 0.59). Average serum M2-PK (U/mL) was 14.75 among stage Ⅲ and 13.10 among stage Ⅰ and Ⅱ CRC patients, about 4 times higher than that among normal people. Average serum M2-PK was 8.58, 6.70, 5.13 and 2.51 U/mL among advanced adenoma, adenomas, non-adenomatous polyps, and inflammatory bowel disease patients, respectively. AUC for serum M2-PK was greater than that for CEA among all colorectal lesions. AUC for serum M2-PK was 0.89 (0.84, 0.94) (95% confidence interval), higher than that for CEA [0.70 (0.62-0.79)] in CRC stage Ⅰ and Ⅱ, 0.89 (0.84-0.94) *vs* 0.73 (0.63-0.83) in CRC stage Ⅲ, 0.81 (0.74-0.86) *vs* 0.63 (0.53 - 0.73) in advanced adenomas, 0.69 (0.64-0.76) *vs* 0.54 (0.47-0.60) in adenomas, and 0.69 (0.62-0.78) *vs* 0.58 (0.48-0.68) in non-adenomatous polyps. The diagnostic sensitivity for all colorectal lesions increased with decrease in the cut-off value of serum M2-PK. The diagnostic sensitivity (%) of serum M2-PK was 100.00 for CRC, 95.12 advanced adenoma, 82.48 adenoma, and 82.98 non-adenomatous polyp. There were no CRC cases missed and 40.51% of

unnecessary colonoscopies were avoided when the cut-off value was 2.00 U/mL.

CONCLUSION: Serum M2-PK can be used as a primary screening test in CRC mass screening. It may be a promising non-invasive biomarker for CRC early detection.

Key words: Serum M2-pyruvate kinase; Colorectal cancer screening; Serum biomarker; Carcinoembryonic antigen

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in men and the second most common in women worldwide[1]. Data from China indicate that CRC incidence is rapidly rising, making it the 2nd-5th most common cancers across different cities[2-4] in the past decades. One of the most important ways to reduce CRC mortality and morbidity is to conduct CRC screening in the population. However, the compliance rate for the immunochemical fecal occult blood test (iFOBT) in a CRC mass screening is not high and is even lower for colonoscopy[5,6].

In order to increase the compliance rate, a screening protocol combining iFOBT with a high risk factors questionnaire (HRFQ) approach as the primary test to screen high risk populations, followed by colonoscopy as a follow-up test to detect CRC and other colorectal diseases, has been recommended by the Department of Disease Prevention and Control, the Ministry of Health of China, for CRC mass screening in China, based on the work of Zheng and her team[7]. This protocol has been used in the China national screening program in the general population in recent years[5,6,8]. The combined HRFQ has improved compliance rate and screening net sensitivity due to the simultaneous screening design and the overall effectiveness of our screening program. However, the overall false positive rate is high, as is the case in all CRC mass screening programs worldwide[8-11].

In our CRC mass screening program, 73.3% (false positive rate from iFOBT or HRFQ) of high risk populations previously underwent unnecessary colonoscopy examinations[6,8]. It is therefore worth further exploring a new simple noninvasive method with high compliance and high sensitivity to identify high risk populations, reduce unnecessary demand for colonoscopies from community residents, and save colonoscopy resources for populations genuinely in need. A serum biomarker with high sensitivity is usually regarded as an ideal primary mass screening test as this is simple, fast, convenient to both participants and clinicians, acceptable to participants, and noninvasive. To date, no effective serum biomarkers can be recommended for CRC mass screening.

We believe that serum tumor M2-pyruvate kinase (M2-PK) can be developed as an effective serum biomarker for CRC mass screening. There are four pyruvate kinase isoforms existing in mammals. The M1 isoform is predominantly expressed in most adult and differentiated tissues; L and R isoforms are expressed in liver and red blood cells; the dimeric form of the M2 isoform is a splice variant of M1 expressed in cancer cells and undifferentiated tissues[12]. Notably it has been reported that tumor tissues exclusively express the embryonic M2 isoform of pyruvate kinase[13,14]. Tumor M2-PK can be detected in blood and fecal samples, probably due to high expression in tumor cells and release into the body fluids[15].

Some studies have reported that fecal M2-PK is a promising biomarker for CRC screening and have recommended fecal M2-PK as a CRC screening marker[16-18]. However, several further studies do not support this view[19-21]. Blood tests are more convenient than fecal tests and can achieve a higher compliance rate in the general population. Clinical studies indicate that serum M2-PK has a higher sensitivity than serum carcinoembryonic antigen (CEA), is a valuable tumor marker in detection of gastrointestinal cancer[22,23] and has advantages in finding localized CRC[24]. No study has investigated the value of serum M2-PK in CRC mass screening in a community setting.

We investigated the potential value of serum M2-PK as a primary test for CRC screening in a community setting and compared its value with serum CEA which is currently one of the most commonly used diagnostic serum biomarkers and still regarded as the best single diagnostic marker for CRC[25,26] due to high specificity. However, serum CEA is not recommended as a screening test for CRC due to low sensitivity[27].

MATERIALS AND METHODS

Study design and population

We conducted a molecular epidemiology study to explore the value of serum M2-PK in CRC mass screening. The study protocol was reviewed and approved by the Institutional Review Board at Zhejiang University Cancer Institute. From July 2006 to December 2008, CRC screening was conducted among community residents aged 40-74 years in Hangzhou City[6,8] following the CRC screening protocol recommended by the China Ministry of Health. All participants gave written informed consent. When participants turned in the signed consent, we collected serum samples from 93 CRC, 41 advanced adenomas, 137 adenomas, 47 non-adenomatous polyps,

Table 1 Basic characteristics of study population for the value of serum pyruvate kinase Isoenzyme M2 and carcinoembryonic antigen in colorectal cancer mass screening in Hangzhou, China, 2006-2008 (mean ± SD)

Colorectal lesion	*n*	Gender		Age (yr)	M2-PK (U/mL)	CEA (ng/mL)
		Male	Female			
Colorectal cancer						
Stage Ⅰ and Ⅱ	55	53	40	59.17 ± 10.71	13.10 ± 12.07	5.74 ± 7.49
Stage Ⅲ	38				14.75 ± 13.39	5.68 ± 5.43
Advanced adenoma	41	25	16	60.17 ± 7.78	8.58 ± 7.65	2.68 ± 1.43
Adenoma	137	68	69	60.34 ± 8.16	6.70 ± 6.97	2.58 ± 3.74
Nonadenomatous polyp	47	25	22	59.04 ± 8.08	5.13 ± 3.73	2.55 ± 2.09
IBD	7	1	6	57.43 ± 7.16	2.51 ± 1.94	1.71 ± 0.91
Normal	158	56	102	57.15 ± 7.96	2.96 ± 2.17	1.98 ± 1.02

IBD: Inflammatory bowel disease; M2-PK: M2-pyruvate kinase; CEA: Carcinoembryonic antigen.

7 inflammatory bowel diseases (IBD), and 158 normal people in the community. According to CRC TNM protocol updated by UICC and AJCC in 2009, among the 93 cases (84 cases from consecutive community patients, 9 cases from our CRC screening site) of CRC, 55 cases were diagnosed as stage 0, Ⅰ and Ⅱ, and 38 cases were diagnosed as stage Ⅲ.

Validation of colorectal lesions

All participants in this study were examined by colonoscopy. If colonoscopy showed a positive result, a biopsy and histopathological diagnosis were carried out after receipt of a signed consent form. Based on the International Classification and guidelines for Colonoscopy Surveillance after Polypectomy[28], CRC was defined as the invasion of malignant cells beyond the muscular mucosa. Patients with intramucosal carcinoma or carcinoma *in situ* were classified as having high-grade dysplasia. Histological classification of total polyps included adenoma (tubular, tubulovillous, or villous) and non-adenomatous polyps. Pathology slides of positive lesions were re-examined and diagnosed by consensus of at least two independent pathologists.

Serum M2-PK determined by enzyme-linked immunosorbent assay

Serum M2-PK was detected strictly in accordance with enzyme-linked immunosorbent assay kit instructions. The test kit measures the dimeric form of tumor M2-PK. The microtiter plates provided in the test kits were pre-coated with antibody specific to tumor M2-PK. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for tumor M2-PK. Our assay carefully followed the instructions of the test kit. Serum CEA was detected automatically by an Abbott i2000SR automatic light meter. Standard serum M2-PK kits (Product ID E0588h) were purchased from Uscn life Science and Technology Company, USA). Serum samples were processed using the following steps: (1) 4 mL elbow vein blood was collected in CRC patients or high risk population under fasting state; (2) the vein blood was kept at under 4 ℃ for 1.5 h, until its natural coagulation; (3) the blood was centrifuged at 3000 r/min centrifugation at 4 ℃ for 5 min; and (4) serum obtained from blood supernatants was again centrifuged for 5 min. Supernatants of serum were removed and placed in Eppendorf tubes and packed immediately in a freezer at -80 ℃.

Statistical analysis

SPSS 16.0 for Windows was used to perform data analysis. Mean ± SD were estimated for serum M2-PK, CEA, and age by colorectal lesion. Linear regressions and t tests were used to compare the serum M2-PK value between gender and age groups. Area under the receiver operating characteristic curve (AUC) and 95% confidence intervals (CI) were estimated for the value of serum M2-PK in diagnosis of CRC, advanced adenomas, adenomas, non-adenomatous polyps, and IBD and compared with serum CEA value. The meaning of AUC is defined as: no diagnostic value if AUC < 0.5; Low diagnostic value if AUC between 0.5-0.7; moderate diagnostic value if AUC between 0.7-0.9; high diagnostic value if AUC > 0.9[29]. The various diagnostic sensitivities and specificities, positive predictive values (PPV) and negative predictive values (NPV), and their 95% CIs were estimated by setting different M2-PK cut-off values for the various colorectal lesions compared with the normal people. The different M2-PK cut-off values were chosen according to the research purposes and scheduled sensitivity and specificity[30].

RESULTS

Table 1 shows the basic characteristics of the study population. The average age was 59.17 ± 10.71 for 93 CRC cases and 57.15 ± 7.96 for 158 normal participants. Among the normal group, there was no significant difference in serum M2-PK between men and women (P = 0.47) or between different age groups (P = 0.59). The average serum M2-PK value in U/mL was 14.75 ± 13.39 among the stage Ⅲ and 13.10 ± 12.07 among the stage Ⅰ and Ⅱ of CRC patients, about 4 fold higher than that (2.96 ± 2.17) among the normal group. The average serum M2-PK value in U/mL was 8.58, 6.70, 5.13, and 2.51 among advanced adenoma, adenomas, nonadenomatous polyps, and IBD, respectively. The average serum CEA value in ng/mL was

Table 2 The area under the receiver operating characteristic curve and 95% confidence interval of serum pyruvate kinase isoenzyme M2 in U/mL and carcinoembryonic antigen in ng/mL in diagnosing colorectal lesions in colorectal cancer mass screening in Hangzhou, China, 2006-2008

Colorectal lesion	Test	AUC	SE	*P*-value	95% CI
CRC stage Ⅰ and Ⅱ	CEA	0.70	0.04	< 0.0001	0.62-0.79
	M2-PK	0.89	0.03	< 0.0001	0.84-0.94
CRC stage Ⅲ	CEA	0.73	0.05	< 0.0001	0.63-0.83
	M2-PK	0.89	0.03	< 0.0001	0.84-0.94
Advanced adenoma	CEA	0.63	0.05	0.01	0.53-0.73
	M2-PK	0.81	0.04	< 0.0001	0.74-0.86
Adenoma	CEA	0.54	0.03	0.28	0.47-0.60
	M2-PK	0.69	0.03	< 0.0001	0.64-0.76
Nonadenomatous polyp	CEA	0.58	0.05	0.09	0.48-0.68
	M2-PK	0.69	0.04	< 0.0001	0.62-0.78
Inflammatory bowel disease	CEA	0.41	0.10	0.40	0.21-0.61
	M2-PK	0.42	0.10	0.40	0.21-0.63

AUC: Area under the receiver operating characteristic curve; CRC: Colorectal cancer; CI: Confidence interval; M2-PK: M2-pyruvate kinase; CEA: Carcinoembryonic antigen.

Table 3 Diagnostic sensitivity and specificity in percentage at 95% confidence interval of serum M2-pyruvate kinase using various cut-off value settings for different colorectal lesions compared with 158 normal people in colorectal cancer mass screening in Hangzhou, 2006-2008 (95% CI)

M2-PK (U/mL)	Colorectal cancer		Advanced adenoma		Adenoma		Non-adenomatous polyp	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
2.00	100.00 (100.00-100.00)	40.51 (32.85-48.16)	95.12 (88.53-100.00)	40.51 (32.85-48.16)	82.48 (76.12-88.85)	40.51 (32.85-48.16)	82.98 (72.23-93.72)	40.51 (32.85-48.16)
2.50	94.62 (90.04-99.21)	55.06 (47.31-62.82)	85.37 (74.55-96.18)	55.06 (47.31-62.82)	71.53 (63.98-79.09)	55.06 (47.31-62.82)	76.60 (64.49-88.70)	55.06 (47.31-62.82)
3.00	91.40 (85.70-97.10)	65.19 (57.76-72.62)	75.61 (62.46-88.75)	65.19 (57.76-72.62)	61.31 (53.16-69.47)	65.19 (57.76-72.62)	65.96 (52.41-79.50)	65.18 (57.76-72.62)
3.50	87.10 (80.28-93.91)	68.99 (61.78-76.20)	73.17 (59.61-86.73)	68.99 (61.78-76.20)	56.93 (48.64-65.23)	68.99 (61.78-76.20)	55.32 (41.11-69.53)	68.99 (61.78-76.20)
4.00	81.72 (73.87-89.58)	74.05 (67.22-80.89)	65.85 (51.34-80.37)	74.05 (67.22-80.89)	49.64 (41.26-58.01)	74.05 (67.22-80.89)	48.94 (34.64-63.23)	74.05 (67.22-80.89)

M2-PK: M2-pyruvate kinase.

5.68 ± 5.43 among stage Ⅲ CRC patients and 5.74 ± 7.49 among stage Ⅰ and Ⅱ, about 2 fold higher than that (1.98 ± 1.02) among the normal group.

The average AUC of serum M2-PK was significantly ($P \leqslant 0.01$) greater than that of CEA among all kinds of colorectal lesions except non-adenomatous polyps (marginal significance, $P = 0.09$) and IBD (no significance, $P = 0.40$), as shown in Table 2. The AUC of serum M2-PK was 0.89 with 95% CI: 0.84-0.94, significantly higher than that of CEA (0.70: 0.62-0.79) for stage Ⅰ and Ⅱ CRC patients, 0.89 (0.84-0.94) *vs* 0.73 (0.63-0.83) for stage Ⅲ CRC, 0.81 (0.74-0.86) *vs* 0.63 (0.53 - 0.73) for advanced adenomas, 0.69 (0.64-0.76) *vs* 0.54 (0.47-0.60) for adenomas, 0.69 (0.62-0.78) *vs* 0.58 (0.48-0.68) for non-adenomatous polyps, and 0.42 (0.21-0.63) *vs* 0.41 (0.21-0.61) for IBD.

The diagnostic sensitivity and specificity with 95% CI of serum M2-PK at different cut-off values are shown in Table 3. When the cut-off value of M2-PK was 2.00 U/mL the sensitivity was 100.00% for CRC, i.e., there were no CRC cases missed. The sensitivity was 95.12%, 81.75%, and 82.98% for advanced adenomas, adenomas, and non-adenomatous polyps (missing rate was 4.88%, 18.25% and 17.02%), respectively. The specificity was 40.51% at the cut-off value of 2.00 U/mL, i.e., a total of 40.51% of unnecessary colonoscopies could be avoided. When the cut-off value increased from 2.00 to 4.00 U/mL, sensitivities of CRC decreased from 100.00% to 81.72% and specificities of CRC increased from 40.51% to 74.05%.

For the comparison of sensitivity and specificity between serum M2-PK and serum CEA in diagnosing positive colorectal lesions, the cut-off value of serum M2-PK was set at 2.00 U/mL and of CEA 5.00 ng/mL. The sensitivity of serum M2-PK was higher but the specificity was lower than that of CEA (Figure 1).

The PPV and NPV with 95% CIs of serum M2-PK with various cut-off value settings for different colorectal lesions compared with 158 normal people in this CRC primary screening are shown in Table 4. The PPV varied from 49.73% to 64.96% and NPV from 100.00% to 87.31% when the cut-off value settings of serum M2-PK were changed from 2.00 to 4.00 U/mL.

Table 4 Positive predictive value and negative predictive value in percentage at 95% confidence interval of serum M2-pyruvate kinase using various cut-off value settings for different colorectal lesions compared with 158 normal people in colorectal cancer mass screening in Hangzhou, China, 2006-2008 (95% CI)

M2-PK (U/mL)	Colorectal cancer		Advanced adenoma		Adenoma		Nonadenomatous polyps	
	PPV	NPV	PPV	NPV	PPV	NPV	PPV	NPV
2.00	49.73 (42.57-56.90)	100.00 (100.00-100.00)	29.32 (21.59-37.06)	96.97 (92.83-100.00)	54.59 (47.81-61.37)	72.73 (63.42-82.03)	29.32 (21.59-37.06)	88.89 (81.63-96.15)
2.50	55.35 (47.62-63.07)	94.57 (89.93-99.20)	33.02 (24.07-41.97)	93.55 (88.56-98.54)	57.99 (50.55-65.43)	69.05 (60.98-77.12)	33.64 (24.69-42.60)	88.78 (82.23-95.03)
3.00	60.71 (52.62-68.80)	92.79 (87.98-97.60)	36.05 (25.90-46.19)	91.15 (85.91-96.39)	60.43 (52.30-68.56)	66.03 (58.59-73.46)	36.05 (25.90-46.19)	86.55 (80.43-92.68)
3.50	62.31 (53.98-70.64)	90.08 (84.76-95.41)	37.97 (27.27-48.68)	90.83 (85.67-96.00)	61.42 (52.95-69.88)	64.88 (57.66-72.10)	34.67 (23.90-45.44)	83.85 (77.52-90.17)
4.00	64.96 (56.31-73.60)	87.31 (67.22-80.89)	39.71 (28.08-51.34)	89.31 (84.02-94.60)	62.39 (53.29-71.48)	62.90 (55.96-69.85)	35.94 (24.18-47.69)	82.98 (76.78-89.18)

M2-PK: M2-pyruvate kinase; PPV: Positive predictive value; NPV: Negative predictive value.

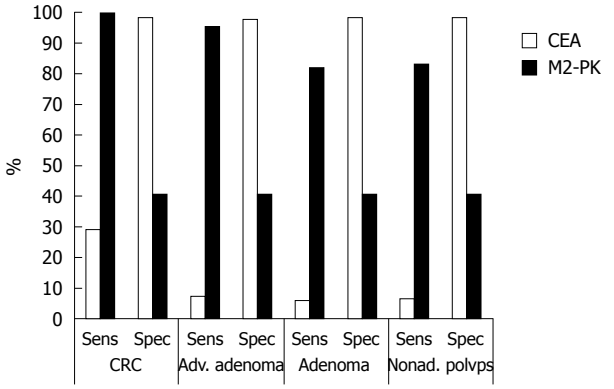

Figure 1 Comparison of diagnostic values between serum M2-pyruvate kinase and carcinoembryonic antigen for positive colorectal lesions based on sensitivity and specificity in Hangzhou, 2006-2008. M2-PK: M2-pyruvate kinase; CEA: Carcinoembryonic antigen; CRC: Colorectal cancer; Adv.: Advanced; Nonad.: Nonadenomatous; Sens: Sensitivity; Spec: Specificity.

DISCUSSION

This study explored the potential value of serum M2-PK in screening CRC and other colorectal lesions in the population and compared its value to that of serum CEA. Overall, the serum M2-PK has a higher diagnostic value than CEA for all types of colorectal lesions except IBD. The serum M2-PK has a moderate to high diagnostic value for early and advanced stages of CRC but CEA has a low to moderate diagnostic value for all stages of CRC. For advanced adenoma the serum M2-PK has a moderate diagnostic value while CEA has a low to moderate value. For both adenoma and non-adenomatous polyps the serum M2-PK has a low to moderate diagnostic value while CEA has a zero to low value. According to this study, both serum M2-PK and CEA have no diagnostic value to IBD. The sensitivity of serum M2-PK is much higher than that of serum CEA in diagnosing all positive colorectal lesions except IBD. The post-hoc statistical power in this study was 100% for all positive colorectal lesions except IBD. Serum M2-PK has the capacity to find more CRC and precancerous lesions than CEA.

We used community patients' samples to test the value of serum M2-PK and found that serum M2-PK has the advantage of detecting earlier stages of CRC. The sensitivity of serum M2-PK for CRC was 100% in this study when the cut-off value was set up at 2.00 U/mL, much higher than that of colonoscopy, iFOBT, and fecal M2-PK[17,31,32]. One of the major goals of CRC mass screening is to reduce mortality through the detection of early-stage CRC, adenocarcinoma and adenoma[31]. A CRC mass screening should avoid missing any CRC cases at the primary stage and confirm the diagnosis at the secondary or later stage of screening, making it possible to achieve the goal of fewer or no deaths from CRC. At this point, a higher-sensitivity screening test is to be preferred to a test with higher specificity in a primary screening. In addition, a serum test avoids the inconvenience of a fecal test and it is simpler, faster, and safer than colonoscopy. Thus, the compliance rate for serum M2-PK in a CRC mass screening is predicted to be higher than that for fecal M2-PK, iFOBT, and colonoscopy. Using serum M2-PK as a primary screening test, the effectiveness of a CRC mass screening should be increased due to high compliance and high sensitivity.

This study showed serum M2-PK is more useful than serum CEA in CRC mass screening because of higher sensitivity and diagnostic value in finding early CRC. The sensitivities of serum CEA were 29.03%, 7.31%, 5.84% and 6.38%, respectively, in diagnosing CRC, advanced adenomas, adenomas, and non-adenomatous polyps, when the serum CEA cut-off value was 5.00 ng/mL. The low sensitivity of serum CEA in detecting early CRC and precancerous lesions limits its application in CRC mass screening.

Adenoma is regarded as a precancerous lesion of CRC. Advanced adenoma is a severe type and defined as adenoma with a diameter of ≥ 10 mm, a villous adenoma, and an adenoma with high grade dysplasia[4,31]. The detection rates of early CRC and advanced adenoma have been used as important indicators in evaluating the effectiveness of a CRC mass screening programs[31]. The projected annual transition rates from advanced adenoma to CRC range from 2.6% to 5.6% among people ≥ 55 years old[33].

Studies show that fecal M2-PK is not a good marker for the detection of colorectal adenomas[34]. Until now, there have been no effective serum biomarkers for finding early CRC and advanced adenomas. Our study indicates that serum M2-PK can obtain a moderate diagnostic value in detecting advanced adenomas, better than that of serum CEA.

Fecal M2-PK can be an indicator of IBD[35,36] and some studies showed plasma M2-PK to have elevated levels in acute and serious inflammation disease[37,38]. However, our study did not find that serum M2-PK is a good index for IBD, for three possible reasons. One is that there were only seven cases of IBD in our study. The second is that the inflammatory process in these seven female patients may be in the early stage, not as severe as those in the other studies. The third possible reason is that there may actually be little difference between IBD cases and normal people. Since there are a considerable number of IBD patients among high risk CRC populations, future research should test the value of serum M2-PK for diagnosing IBD in a large study population.

The findings that serum M2-PK among normal people is low and not influenced by age and gender in this study are expected. Tumor M2-PK is an enzyme within tumor metabolism. The serum level of tumor M2-PK among normal people should be low compared to that among colorectal lesion patients and should not vary by gender and age. The average level of serum M2-PK among 158 normal people was 2.96 U/mL which is much lower than those in clinical patients or volunteers[22,23]. Our community-based results for serum levels of M2-PK associated with the TNM Classification of Malignant Tumors and Duke's staging in CRC are supported by these patient-based clinical studies[22,23]. Because our result for normal serum level of M2-PK was based on a large sample size (158) from communities in a CRC mass screening program, it is reliable and can be generalizable.

Serum M2-PK with high sensitivity can achieve moderate to high diagnostic value in detecting early CRC and advanced adenomas and is superior to serum CEA. It also plays an important role in reducing costs, inconvenience, and colonoscopy-related complications during CRC screening. In addition, the compliance rate for serum M2-PK should be improved compared to other tests in a mass screening program. Thus the effectiveness of CRC mass screening programs should be improved greatly. In the long run, the healthcare burden from CRC should be minimized due to low CRC incidence and mortality in the community, the desired outcome of a successful CRC screening program.

Overall, we conclude that serum M2-PK can be used as an efficient primary screening test for CRC mass screening. It is simpler and faster than a fecal test and cheaper, more convenient, and safer than colonoscopy. It is a promising non-invasive biomarker for CRC early detection. We will test its value in other community settings and or in a large study population in the future.

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COMMENTS

Background

Colorectal cancer (CRC) has become a big burden to global health over the past decades. Mass screening is an effective way to reduce CRC mortality and incidence in the population. However, the low compliance for current screening tests affects the effectiveness of CRC mass screening programs. A serum test avoids the inconvenience of a fecal test and it is simpler, faster, and safer than colonoscopy. Therefore, a serum test can obtain a higher compliance for CRC screening in the general population than a fecal test or colonoscopy. A serum biomarker test with high sensitivity is intuitively an ideal test for CRC mass screening. To date, no effective serum biomarkers can be recommended for CRC mass screening.

Research frontiers

M2 isoform of pyruvate kinase (M2-PK) is a splice variant of M1 and expressed in cancer cells and undifferentiated tissues. The dimeric form of M2-PK is termed tumor M2-PK. Clinical research shows tumor M2-PK is associated with the TNM Classification of Malignant Tumors and Duke's staging in CRC. The authors hypothesized that serum tumor M2-PK can be developed as an efficient primary screening test in CRC screening in the population. No previous study has investigated the value of serum tumor M2-PK in CRC mass screening in a community setting.

Innovations and breakthroughs

The level of serum M2-PK among normal people was low and not affected by gender and age. Serum M2-PK among CRC patients was about 4 fold higher than that among the normal. Serum M2-PK has moderate value in diagnosing CRC and advanced adenoma. The diagnostic sensitivity of serum M2-PK was 100.0% for CRC, i.e., there were no CRC cases missed, and 40.5% of unnecessary colonoscopies avoided when the cut-off value was 2.00 U/mL. This is the first study that has investigated the value of serum M2-PK in CRC mass screening in a community setting.

Applications

Results from this study suggest that serum M2-PK can be used as a primary screening test in CRC mass screening due to its high sensitivity and high compliance. It is a promising non-invasive biomarker for CRC early detection.

Terminology

Advanced adenoma is a severe type of adenoma and defined as adenoma with a diameter of $\geqslant$ 10 mm, a villous adenoma, and an adenoma with high grade dysplasia.

Peer review

The author investigated the potential value of serum M2-PK as a promising non-invasive biomarker for CRC mass screening, due to lower sensitivity of serum CEA for CRC screening. Positive results had been achieved in this study.

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CASE REPORT

Bone lesions in recurrent glucagonoma: A case report and review of literature

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Abstract

Glucagonomas are rare neuroendocrine tumors that arise from α cells of the pancreatic islets. Most of them are malignant and usually present as metastatic disease. Sites most commonly involved in metastases are the liver and regional lymph nodes. Bone metastases are rare events and only a few cases have been reported in the literature. We present the case of a 53-year-old male with a medical history of recurrent non-functioning glucagonoma. He presented 17 years after the initial diagnosis with new blastic bone lesions involving the T1 vertebra and the sacrum. Diagnostic steps and medical management in metastatic glucagonoma are also reviewed.

Key words: Glucagonoma; Bone metastases; Blastic lesion; Octreoscan

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INTRODUCTION

Neuroendocrine tumors of the pancreas are rare malignancies, accounting for 1%-2% of pancreatic neoplasms. Also known as islet cell tumors, neoplasms in this heterogeneous group have distinct histological and biological behavior and are now believed to arise from multipotent stem cells located in the ductal epithelium[1]. From a clinical point of view, these tumors are classified into functioning and non-functioning. Functioning tumors are neoplasms that secrete inappropriate amounts of hormones causing clinical endocrinopathy. The most common secreting types are insulinoma and gastrinoma[2].

Glucagonomas are neuroendocrine tumors that arise from α cells of the pancreatic islets[3]. They present as encapsulated firm nodules that reach 25 cm in diameter and usually occur in the tail of the pancreas. Histologically, glucagonoma consist of cords and nests of well-differentiated islet cells. Nevertheless, despite their benign appearance, most glucagonomas are malignant and the disease is usually metastatic at diagnosis[3,4]. Metastatic disease usually involves the liver and lymph nodes and rarely extends to the bones. Therefore, only a few cases of glu-

cagonomas with bone metastases have been reported in the literature. These bone metastases are mostly spinal[3-6]. We present the case of a male patient with a history of recurrent nonfunctioning glucagonoma who was found to have blastic bone lesions.

CASE REPORT

A 53-year-old male presented with left upper extremity numbness and weakness. His medical history revealed that he had been diagnosed with glucagonoma at age 36. More specifically, in April 1993, the patient experienced sudden epigastric pain radiating to the left upper quadrant. An abdominal ultrasound revealed a 5-6 cm mass located between the body of the pancreas and the anterior wall of the stomach and the patient underwent exploratory laparatomy converted into a distal pancreatectomy. Histology showed an islet cell tumor positive for glucagons, chromogranin and synaptophysin and negative for somatostatin, gastrin, insulin and pancreatic polypeptide, leading to the diagnosis of a non-functioning glucagonoma. His condition remained stable until February 1998 when an abdominal computed tomography (CT) scan revealed a recurrent mass in the mid-portion of the pancreas (6 cm × 8 cm × 6 cm). The tumor was surgically resected and histology confirmed the recurrence of the neuroendocrine tumor. The patient was on regular follow-up thereafter with serial CT scans of the abdomen and pelvis.

Seventeen years after the initial diagnosis, the patient presented to our clinic with left hand numbness and weakness radiating under the left arm and left axilla. The symptoms had started 2 mo before. He also noticed weakness in his left upper extremity but denied any pain. The patient experienced no other symptoms and review of the systems revealed weight gain, good appetite and performance status. Complete physical examination was unremarkable with no evidence of skin lesions, rashes, lymphadenopathy or focal neurological deficits. He underwent a chest CT scan that revealed a 20 mm blastic lesion suspicious of metastasis in the left transverse process of the T1 vertebra (Figure 1A). Additional blastic lesions were found on the posterior aspect of the right fifth rib and in both transverse processes of the S1 vertebra (measuring 3 mm, 15 mm and 15 mm in diameter, respectively) (Figure 1B and C). The magnetic resonance imaging (MRI) scan of the spine showed non-enhancing foci of low signal intensity in the bilateral sacrum, in accordance with the sclerotic lesions seen on prior CT scan. Bone scan showed no foci of abnormal increased activity. Octreotide scan did not show increased uptake in the area of bone lesions but showed focal activity in the surgical bed, raising the suspicion of recurrent disease. Although the diagnosis of osteosclerosis was part of the differential, the above mentioned lesions were not noticed in a similar study conducted 2 years before, suggesting that these lesions were metastatic. Chromogranin A (ChA) was found mildly elevated (48 ng/mL-normal values < 36.4 ng/mL) whereas glucagon, serotonin and gastrin levels were within the normal range. The patient moved to a different state and continued treatment there. The bone biopsy that had been scheduled was never performed.

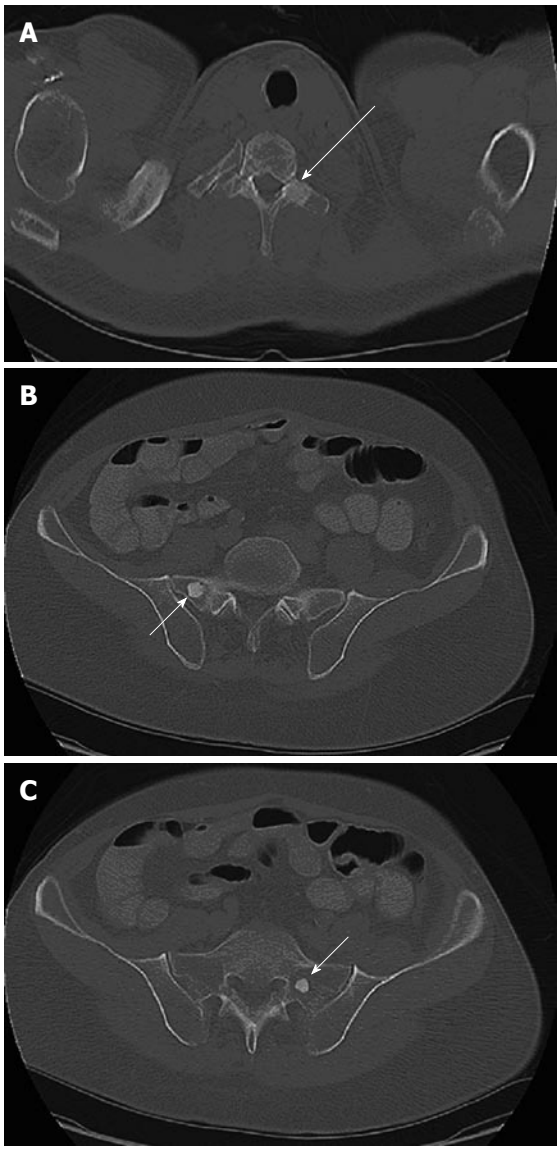

Figure 1 Axial computed tomography scan of the patient. A: Computed tomography (CT) through the upper chest displayed using the bone window settings. There is a 2-cm well-defined sclerotic lesion within the left transverse process of the T1 vertebra (arrow); B: CT through the pelvis displayed using the bone window settings. There is a 1.5-cm, well defined, sclerotic lesion (arrow) in the right sacrum adjacent to the sacroiliac joint; C: CT through the pelvis displayed using the bone window settings. There is a 1.5-cm, well defined, sclerotic lesion (arrow) in the left sacrum.

DISCUSSION

The diagnosis of glucagonoma includes: characteristic clinical features, elevated hormone levels, imaging findings and histological confirmation. The presentation of glucagonomas has been associated with necrolytic migratory erythema, diabetes mellitus, anemia, weight loss, di-

arrhea, deep venous thrombosis, neuropsychiatric symptoms and hypoaminoacidemia[4,6,7]. The main features of glucagonoma syndrome include hyperglycemia, increased muscle catabolism with wasting and cutaneous manifestations associated with necrolytic migratory erythema. Glucagon secretion is responsible for most of the observed signs and symptoms[6,8]. The endocrine manifestations are more common in advanced stages and may be related directly to tumor size, but lack of clinically important secretory activity can be observed in non-functioning tumors even in widely metastatic disease[9]. This was also the case in our patient who had no clinical manifestations of glucagonoma syndrome at initial presentation or later.

Laboratory abnormalities in functioning neoplasms include hyperglucagonemia and hyperglycemia. Glucagon levels are usually above 1000 pg/mL although in some glucagonomas, levels do not exceed the upper normal range[10]. Hormones not directly involved in the clinical syndrome may also be elevated: ChA and pancreatic peptide (PP) levels are raised in 50%-80% of cases, including nonsecretory tumors. A combined assessment of PP and ChA is particulary useful for the diagnosis of nonfunctioning cases and their increased levels also seem to correlate with overall disease burden[10]. Besides their role as tumor markers, they can also be utilized to monitor the therapeutic response[11]. In our case, glucagon levels were reported normal with only a mild increase in ChA levels.

The natural history of this malignancy reveals that the prevalence of metastatic disease at time of diagnosis varies from 50% to 100%[12]. Common metastatic sites are the liver and regional lymph nodes. Other reported sites for metastatic glucagonoma are adrenal glands, kidneys and lungs[6]. Bone metastases are rare events with only seven cases reported in the literature to date[3,13,14]. In most of these cases, vertebral metastases were described. In one of these cases, bone metastases were the initial finding of glucagonoma[13] and spinal cord compression was observed in another patient[3]. There has also been a case of misdiagnosis where the bone scan showed abnormality in the proximal femur, initially considered avascular necrosis[14]. Bone metastases from pancreatic islet carcinoma have also been reported in dogs[15]. Our patient had blastic lesions involving the vertebrae and the sacrum. Unfortunately, the patient moved to another state before bone biopsy was performed. However, the radiological findings deserve attention and clinicians should be aware of this rare site of glucagonoma metastases.

Octreotide scan has become one of the most important tools in the initial diagnosis and staging of these tumors. CT, MRI scans, endoscopic or perioperative ultrasonography are used for diagnosis and also for evaluation of response to treatment[16]. Arterial stimulation and venous sampling with calcium loading also seems to be an effective, although more invasive, method of detecting glucagonomas[17].

Somatostatin receptor scintigraphy using radiolabeled octreotide is an important diagnostic tool as glucagonomas express somatostatin receptors in more than 80% of cases. Due to the rarity of these neoplasms, the sensitivity and specificity of this imaging technique has not been clearly established. Possible causes of false-negative results are high levels of endogenous somatostatin competing for receptors with the radiolabeled octreotide or causing receptors downregulation. Absence or minimal expression of one of the somatostatin receptor subtypes (type 2) can also lead to poor visualization since this receptor holds the highest affinity for octreotide[5]. The octreoscan in our patient failed to show increased bone uptake but did show some uptake in the surgical bed, suggesting possible recurrent disease. As shown above, this study cannot rule out metastatic glucagonoma. Unfortunately, our patient did not undergo biopsy. However, the radiological findings deserve attention to make clinicians aware of this rare site of metastases of glucagonoma.

Regarding prognosis, glucagonomas are slowly growing tumors usually advanced by the time of diagnosis. When the primary tumor can be controlled, aggressive radical surgery and complete tumor resection offer long-term survival[18,19]. Once glucagonoma is metastatic, cure is rarely achieved.

Treatment of the glucagonomas with metastatic involvement other than the liver alone consists of targeting excessive hormonal secretion and tumor growth. Somatostatin analogues such as octreotide are highly effective in controlling symptoms related to glucagon hypersecretion[20]. No benefit in non-secreating tumors has been shown, as there is no documented antitumoral activity of octreotide. Interferon-alfa also improves symptoms in up to 50% of patients with pancreatic endocrine tumors. Multiple cytotoxic drugs have been used, mostly combinations of streptozocin with doxorubicin or fluorouracil. Cisplatin and etoposide have been used in rapidly progressive tumors[21]. Other studies have examined the role of topotecan, oxaliplatin, gemcitabine, capecitabine and temozolomide-based regimens in the treatment of neuroendocrine tumors of the gastrointestinal tract[22,23]. Unfortunately, the benefit of current regimens remains modest considering the poor tumor response and increased toxicity[6]. New molecularly targeted therapeutic options are under investigation. VEGF pathway inhibitors, such as sunitinib and bevacizumab have shown promise in delaying progression of metastatic pancreatic endocrine neoplasms. Inhibition of mTOR, using temsirolimus and everolimus, has also been studied[23]. Radioembolization with selective internal radiation microspheres in cases of liver metastases can achieve relatively long-term response[24]. Finally, external beam radiotherapy is used as palliative care in bone metastases or bulky disease[23].

In conclusion, glucagonomas are rare pancreatic endocrine tumors. By the time of diagnosis, more then half of these tumors are already metastatic. Bone metastases are rare in glucagonomas with only 7 other cases reported in the literature. Laboratory and imaging findings can be inconclusive, especially in case of non-secretory types. Even octreotide scan may lack sensitivity, depending on the somatostatin receptor profile and/or somatostatin

endogenous secretion. In confirmed cases of bone metastases, therapy should include a systemic approach using chemotherapy combinations along with molecularly targeted therapy. Glucagonomas expressing somatostatin receptors 2 and 5 may benefit from radiolabelled somatostatin therapy. External beam radiation can be used palliatively.

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Events Calendar 2012

January 14-17, 2012
10th Oncology Controversies and Advances Update
Steamboat Springs,
CO, United States

January 19-21, 2012
EASL Monothematic Conference: IMLI - Immune Mediated Liver Injury
Birmingham, United Kingdom

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL, United States

February 2-4, 2012
2012 Genitourinary Cancers Symposium
San Francisco, CA, United States

February 6-8, 2012
Pediatric Cancer Translational Genomics
Phoenix, AZ, United States

February 8–10, 2012
The 84th Annual Meeting of Japanese Gastric Cancer Association
Osaka, Japan

February 10-11, 2012
Cancer Survivorship for Clinicians
Seattle, WA, United States

February 14-17, 2012
ASCO Multidisciplinary Cancer Management Course
Eldoret, Kenya

February 20-24, 2012
Word Conference on Colorectal Cancer
FL, United States

February 22-23, 2012
National Cancer Institute Annual Biospecimen Research Network Symposium: "Advancing Cancer Research Through Biospecimen Science"
Bethesda, MD, United States

February 22-25, 2012
30th German Cancer Congress
Berlin, Germany

February 24, 2012
ASCO-German Cancer Society Joint Symposium, German Cancer Congress
Berlin, Germany

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 7-8, 2012
First International Gulf Joint Conference: Management of colon, breast, and lung cancer (Joint Symposium)
Dammam, Saudi Arabia

March 9-10, 2012
ESMO Conference on Sarcoma and GIST
Milan, Italy

March 10-11, 2012
Colorectal Polyps and Cancers: A Multidisciplinary Approach
Scottsdale, AZ, United States

March 17-21, 2012
Methods in Cancer Research Workshop (Advanced Cancer Course)
Al Asha, Saudi Arabia

March 22-24, 2012
The 1st St.Gallen EORTC Gastrointestinal Cancer Conference
St.Gallen, Switzerland

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA, United States

April 20–21, 2012
OOTR 8th Annual Conference - Organisation for Oncology and Translational Research
Kyoto, Japan

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 19-22, 2012
Digestive Disease Week 2012
San Diego, CA, United States

June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
Lake Tahoe, NV, United States

June 27-30, 2012
ESMO 14th World Congress on Gastrointestinal Cancer 2012
International Convention Center Of Barcelona,
Barcelona, Italy

July 1–5, 2012
10th World Congress of the International Hepato-Pancreato-Biliary Association
Paris, France

July 5-7, 2012
International Research Conference on Liver Cancer
Heidelberg, Germany

July 6-8, 2012
The 3rd Asia – Pacific Primary Liver Cancer Expert Meeting "A Bridge to a Consensus on HCC Management"
Shanghai, China

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 14-16, 2012
ILCA 2012 - Sixth Annual Conference of the International Liver Cancer Association
Berlin, Germany

September 21-22, 2012
Research Symposium, Inflammation and Cancer
Houston, TX, United States

October 15 - 17 2012
13th World Congress of the International Society for Diseases of the Esophagus
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December 5-8, 2012
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Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc*. Arabic numerals such as 23, 243, 641 should be read 23243641.

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Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
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