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Recent developments in the research on biomarkers of cholangiocarcinoma in primary sclerosing cholangitis

Wannhoff Andreas, Gotthardt Daniel N.*

Department of Internal Medicine IV, University Hospital Heidelberg, Heidelberg, Germany

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Summary Primary sclerosing cholangitis (PSC) is characterized by a chronic inflammatory process of the bile ducts of unclear aetiology. It is often complicated by cholangiocarcinoma (CCA) with a dismal prognosis. Early detection of CCA is important because treatment options for advanced disease are limited. Besides the established markers, like CA19-9, recent developments have been made using latest technologies. This review summarizes the recent advances and remaining limitations of biomarkers of CCA in PSC.

Introduction

Primary sclerosing cholangitis (PSC) is a chronic inflammatory disease of both intra-hepatic and extra-hepatic bile ducts. Due to disease progression, it might lead to formation of biliary strictures and eventually to cirrhosis and hepatic decompensation [1]. Besides this, patients with PSC have a high risk of developing malignant complications, with cancer as the most prominent cause of death in these patients; in fact, the risk for developing biliary tract cancers, particularly cholangiocarcinoma (CCA), is increased approximately 160 times. In patients with concomitant inflammatory bowel disease (IBD), the risk for developing colorectal cancer is also increased [2,3]. Regular ultrasound examinations of the gallbladder and yearly colonoscopies in patients with IBD help to identify premalignant lesions, whereas screening and diagnosis of CCA are more difficult [4]. CCA has a poor prognosis, with an annual incidence of 0.5%—1.5% in patients with PSC [5,6]. As recently shown in a large population-based study, the risk for CCA in the first year after diagnosis is only 2% and thereby lower than previously expected [7]. However, the cumulative risk increased to 20% after a disease course of 30 years. Thus, regular monitoring of these patients is clinically needed.

Screening for hepatobiliary malignancies in PSC patients has the potential to improve the patients' outcome, including survival, as indicated by a recent study [8]. Methods that might be used in diagnosing or screening for CCA in patients with PSC are biomarkers, endoscopy combined with or without histocytopathology, and imaging modalities [4].

In this review, we will summarize and evaluate the latest developments in biomarker research in PSC-associated CCA (PSC-CCA).

* Corresponding author. Mediteo GmbH, Haupstrasse 90, 69117 Heidelberg, Germany.
E-mail address: daniel.gotthardt@gotthardt.com (D.N. Gotthardt).

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Table 1 Diagnostic performance of CA19-9 and CEA. Results for the diagnostic performance for CA19-9 and CEA reported from studies included in this review. The study of CA19-9 investigated the use of genotypes for *fucosyltransferases* to improve the performance of CA19-9. The authors identified three groups of patients with distinct CA19-9 serum levels (termed low, intermediate, and high CA19-9 biosynthesis activities). Patients with a low biosynthesis activity are not included in the table because these patients do not express CA19-9. The same group also investigated the association of the *fucosyltranferase 2* genotype (variant rs610338) with CEA serum levels.

Parameter	N (PSC/PSC-CCA)	AUROC	Sens. (%)	Spec. (%)	Ref.
CA19-9	392/41[a]	0.86	78	90	[10]
Patients with intermediate CA19-9 biosynthesis activity	267/34	0.90	82	90	
Patients with high CA19-9 biosynthesis activity	97/4	0.97	100	88	
CEA	205/19[b]	0.67	37	91	[17]
GG genotype in *FUT2* variant *rs601338*	n.r.	0.73	63	88	
GA genotype in *FUT2* variant *rs601338*	n.r.	0.61	71	62	
AA genotype in *FUT2* variant *rs601338*	n.r.	0.82	75	100	

AUROC: area under the receiver operating characteristic curve; Sens.: sensitivity; Spec.: specificity; Ref.: reference; n.r.: not reported.
[a] Of 41 patients with biliary tract cancer included in the study, 36 and 5 had CCA and gallbladder cancer, respectively.
[b] Of 19 patients with biliary tract cancer included in the study, 15 and 4 had CCA and gallbladder cancer, respectively.

Carbohydrate antigen CA19-9 and carcinoembryonic antigen

CA19-9 is probably the most commonly used tumor marker in PSC patients. However, the interpretation of CA19-9 measurements can be very challenging. This is at least in part due to the limitations of studies on CA19-9 in PSC and consequently current international guidelines on recommendations regarding CA19-9 for diagnosis or screening of CCA in PSC are vague [1,9]. Only one prospective study had been performed on this topic, which however was limited by the occurrence of only three CCAs during follow-up. The previously performed retrospective studies were either small in size or resulted in very different CA19-9 cut-off values for diagnosis of CCA.

While not all of these limitations have been overcome in the last years, recent studies provided interesting data that at least can provide some useful help in the interpretation of CA19-9. The largest and most prominent of these studies identified the genotype of *fucosyltransferase* (*FUT*) 2 and 3 to significantly influence the serum levels of CA19-9 in patients with PSC. These genes encode for two proteins also known as the Secretor and Lewis enzyme. They not only determine an individual's Lewis blood group, but also catalyse the final steps of CA19-9 biosynthesis. Based upon the *FUT* genotype, the study identified groups with low, intermediate, and high CA19-9 biosynthesis levels that showed distinct CA19-9 serum levels [10]: Median CA19-9 serum levels were 2.0 U/mL, 17.0 U/mL, and 37.0 U/L respectively. Similar results were also found regarding pancreatic cancer [11,12]. Patients with a low biosynthesis genotype — which most likely will not benefit from CA19-9 as a screening marker, since they are genetically incapable of CA19-9 synthesis — can also be identified by a Lewis-negative blood group.

False-positive test results for CA19-9 are frequently found and limit the use of CA19-9 as a screening parameter, because approximately 1/3 of the patients with an increased CA19-9 > 129 U/mL, which was identified as the optimal cut-off in another large study [13], were not diagnosed with CCA [14]. Furthermore, in approximately 1/3 of these patients without CCA and with increased CA19-9, no reason for CA19-9 elevation could be identified in two different studies [14,15]. Interestingly, the use of *FUT* genotype-dependent cut-off values resulted in a 43% reduction of these false-positive test results in the previously mentioned study [10]. It thus can be speculated that increased CA19-9 serum levels might be due to the individual's *FUT* genotype in at least some of the cases.

One possible explanation for the increased CA19-9 in PSC patients without CCA is bacterial cholangitis [14]. It causes increased CA19-9 levels and limits the use of CA19-9 for CCA screening. In contrast, the presence of dominant strictures was identified to influence CA19-9 serum levels to a much lesser extent and to not affect CCA diagnosis [16].

In a further study, an association between *FUT* genotype and CEA was also found [17]. That study identified a significant influence of *FUT2* genotype on CEA serum levels, which was most prominent in a subgroup genetically incapable of CA19-9 synthesis. CEA additionally is not influenced by the presence of bacterial cholangitis [18]. Whether this might be a possible clinical setting wherein CEA is superior to CA19-9 has to be evaluated in future studies. Thus, the combination of *FUT* genotyping and measurement of CA19-9 and CEA levels might be interesting with regard to future CCA screening in PSC. Results for the area under the curve (AUC), sensitivity, and specificity of the studies analysing *FUT* genotype and CA19-9 or CEA are summarized in Table 1. Even though the results have not yet been validated in a second study, they are supported by findings from a previous published genome-wide association study [19].

In the case of equivocal results for brush cytology (including atypical and suspicious findings) obtained during ERCP, serum CA19-9 was not found to be an independent predictor for malignancy. In contrast, only suspicious cytology results (excluding atypical findings) in combination with increased CA19-9 (> 129 U/mL) was almost diagnostic for CCA [20].

Even though these results have not been validated in further studies, with regard to clinical practice, at least some guidance on the interpretation of CA19-9 can be given: First of all, it should not be measured during episodes of bac-

Table 2 Serum biomarkers for diagnosis of CCA. The area under the receiver operating characteristic curve (AUROC), sensitivity (Sens.), and specificity (Spec.) for serum biomarkers of CCA are summarized. Results are reported separately for studies focusing on PSC and PSC-CCA and for studies investigating the difference between PSC and CCA.

Parameter	PSC vs. PSC-CCA				PSC vs. CCA				Ref.
	N (PSC/PSC-CCA)	AUROC	Sens. (%)	Spec. (%)	N (PSC/CCA)	AUROC	Sens. (%)	Spec. (%)	
CYFRA 21-1	19/6	n.r.	56	88					[21]
Angiopoietin-2					68/28[a]	0.85	74	94	[22]
Fucosylated Fetuin-A					39/39	0.81	62	90	[24]
miR-222 and miR-483-5p					40/40	0.77	n.r.	n.r.	[25]

Ref.: reference; n.r.: not reported.
[a] One hundred and two patients with benign biliary disorder were included for the calculation of these values.

terial cholangitis. Further, in cases of markedly elevated CA19-9, the FUT genotype can aid in the interpretation, and should raise suspicion of CCA in patients not having a high CA19-9 biosynthesis genotype. With regard to future developments, the authors of this review just recently submitted a manuscript for publication on serial CA19-9 measurements that will provide further help for the interpretation of CA19-9 measurements and the interval of CA19-9 measurement.

Other serum biomarkers

Even though several attempts, which are summarised in Table 2, to investigate serum biomarkers of CCA in PSC patients have recently been undertaken, the value with regard to cancer screening or diagnosis of CCA in PSC patients is very limited in almost all studies. Only two of the studies actually reported results for PSC compared to PSC-CCA patients, which however were rather disappointing.

The first was a small study including patients with PSC ($n = 19$) and PSC-CCA ($n = 6$) and investigated the biomarker CYFRA 21-1, which has previously been evaluated as a tumour marker in other malignancies. The authors reported sensitivities and specificities of 56% and 88% at a cut-off of 1.5 ng/mL and of 17% and 95% at 3.0 ng/mL, respectively. Nevertheless, the use of CYFRA 21-1 as a screening parameter is limited because the authors did not find a significant difference in its serum levels between PSC and PSC-CCA [21]. The second study investigated angiopoetin-2, which was proposed to play a pro-angiogenetic role in CCA development. Even though, angiopoetin-2 serum levels were increased in patients with CCA, the comparison of patients with PSC and PSC-CCA did not result in significant differences [22].

Of the studies that included CCA and not-PSC-CCA patients only, the one by Arbelaiz et al. seems most promising. The authors isolated extra-cellular vesicles from the serum of patients with PSC, CCA, HCC, and healthy controls and performed proteomic analysis of these vesicles. Extra-cellular vesicles are lipid bilayer spheres of 40 to > 1000 nm generated by diverse cell types and contain specific proteins, lipids, RNA species, deoxyribonucleic acid, and metabolites. Fibrinogen gamma chain, alpha-1-acid glycoprotein 1, and S100A8 proteins showed the best diagnostic capacity, with AUC values of 0.796, 0.794, and 0.759, respectively. Most interestingly, further identified peptides showed an improved diagnostic capacity in patients with CCA in stage I + II compared with those with PSC. These proteins included fucolin 2, inter-alpha-trypsin inhibitor heavy chain H4, and fibrinogen gamma chain, which had AUC values of 0.956, 0.881, and 0.881, respectively. Even though the results of the study are very impressive, they yet need to be validated in further studies and especially in patients with PSC-CCA. Further, the isolation and analysis of extracellular vesicles might limit widespread use of this approach at the current time.

The further studies did not report on PSC-CCA and were further limited by diagnostic capacities, measured by AUC in ROC analysis that did not exceed that of CA19-9, thus providing little benefit to current clinical practice.

The serum levels of IgG4 were found to be not associated with an increased risk of CCA in a study on 345 patients with PSC. In the same study, a history of pancreatitis was associated with increased IgG4 levels, which additionally might indicate the presence of IgG4-associated cholangitis instead of PSC [23]. Betesh et al. analysed the levels of cacodylate proteins in patients with PSC and CCA. They found that serum levels of fucosylated fetuin-A had the best ability to differentiate CCA from PSC, with an AUC in ROC analysis of 0.81. The study included 39 patients each with PSC as well as with CCA without PSC [24]. The differential expression of micro-ribonucleic acids (miRNA/miR) in serum samples was analysed by Bernuzzi et al. After the discovery phase, the authors also performed validation in a second cohort, which included 40 healthy controls, 40 patients with PSC, 40 patients with CCA, and 20 patients with primary biliary cholangitis. The results revealed a difference in the expression of miR-222 and miR-483-5p when comparing patients with PSC with those with CCA. The AUC for the combined used of these two miRNAs however was only 0.77 [25].

Other studies mainly reported results that might help to stratify PSC patients according to their risk of CCA development. These biomarkers alone do not allow diagnosis of CCA but could help to detect patients at increased risk of PSC and thereby possibly increase diagnostic performance of other biomarkers. Recently, anti-GP2 IgA was identified as an interesting new biomarker in PSC, which was associated with a worse outcome, which was mainly due to an increased rate of CCA in patients positive for anti-GP2 IgA. Although no difference was found between PSC and PSC-CCA in that study, the odds ratio for developing CCA was 5.0 [26]. Several recent studies investigated a reduction or

Table 3 Markers of CCA identified in bile. The diagnostic performance of biliary biomarkers for differentiation of PSC from CCA on top of PSC (PSC-CCA) and from not-PSC-associated CCA is reported.

Parameter	PSC vs. PSC-CCA				PSC vs. CCA				Ref.
	N (PSC/PSC-CCA)	AUROC	Sens. (%)	Spec. (%)	N (PSC/CCA)	AUROC	Sens. (%)	Spec. (%)	
RNU2-1f	11/12	0.86	67	91					[39]
miR-412	52%12	0.81	50	89					[52]
miR-640		0.81	50	92					
miR-1532		0.78	67	90					
miR-3189		0.80	67	89					
Combination of miR-1537 with CA19-9		0.91	73	93					
Combination of 5 miRNAs extracted from biliary extra-cellular vesicles					13/46[a]	n.r.	67	97	[38]
Fucolin 2					30/13[c]	0.96	100	81	[53]
Inter-alpha- trypsin inhibitor heavy chain H4[b]					30/13[c]	0.89	92	81	
Fibrinogen gamma chain[b]					30/13[c]	0.89	92	81	

AUROC: area under the receiver operating characteristic curve; Sens.: sensitivity; Spec.: specificity; Ref.: reference; n.r.: not reported.
[a] Fifty patients with benign biliary disorder were included for calculation of these values.
[b] Identified by proteomic analysis of biliary extra-cellular vesicles.
[c] Comparing PSC with CCA in UICC stages I + II only.

normalization of serum alkaline phosphatase (ALP) as predictive marker in patients with PSC. The risk of developing CCA was significantly higher in those patients that did not experience a reduction of ALP. [27—31]. Similar results were reported in two further studies that found an increased risk of developing CCA in patients with worse liver function tests [32,33] In a large, retrospective multicentre study the CCA risk was further related to the age at PSC diagnosis, which could as well be confirmed in a smaller single-centre study [34,35].

Biomarkers measured in bile

After serum, bile is the second-most used body fluid in the research of CCA biomarkers. Bile is in direct contact with the cholangiocytes and can be obtained for analysis during endoscopic retrograde cholangiography (ERC). Unfortunately, most of these studies were limited by the inclusion of heterogeneous groups of patients, including patients with PSC, other benign biliary disorders (e.g., gallstone disease), CCA (mostly without PSC), and other malignancies. A summary of the studies is provided in Table 3.

One of the studies that focused on patients with PSC and PSC-CCA identified biliary calprotectin as potential biomarker. Its biliary levels were significantly higher in PSC without CCA compared with PSC-CCA. In contrast to some much smaller studies included in this review, 56 and 13 patients with PSC and PSC-CCA, respectively, were included in that study. Calprotectin in bile was significantly lower in patients with PSC-CCA, but no information on sensitivity or specificity was presented [36]. Given the quality of the study, this approach warrants further investigation. A second study focusing on patients with PSC and PSC-CCA found no association of IgG ANCA positivity in bile with development of CCA during follow-up [37] , which thus cannot be recommended. Lower bile levels of patients with PSC compared with patients with PSC-CCA were reported for several different miRNAs. ROC analysis revealed AUC between 0.78 and 0.81 for these miRNAs, which however was lower than that for CA19-9 alone in the same study (AUC: 0.88) [36]. Extraction of miRNA from biliary extra-cellular vesicles was performed by Li et al. The study included 46 patients with CCA, including 4 patients with PSC-CCA, and 50 control patients, including 13 patients with PSC. The results revealed sensitivity and specificity of 67% and 96%, respectively, for a model that included biliary levels of five different miRNAs (miR-191, miR-486-3p, miR-1274b, miR-16, and mi-R484) [38]. Besides the heterogeneity of the study population, the complexity of the approach might further limit its use.

Further studies on biliary biomarkers were performed, but these studies did not explicitly focus on the difference between PSC and PSC-CCA, but included patients with CCA not on top of PSC as well. These studies identified higher biliary levels of U2 small nuclear RNA fragments and reduced bile phosphatidylcholine levels in patients with CCA compared with those with PSC [39,40]. To which extend results apply to PSC-CCA as well cannot be estimated.

Interestingly, in two studies, biliary microorganisms were associated with an increased risk of developing CCA in PSC. These included the presence of biliary candidiasis and reduced microbial diversity [41,42]. This might help identify patients at increased risk of developing CCA with need for closer surveillance.

All of these results have not yet been validated. Further, all these analyses require an ERC for bile fluid collection. However, given the known side effects and the risk of cholangitis after ERC in PSC patients, it does neither seem justified nor feasible to screen for CCA with any of these biomarkers outside clinical studies.

Table 4 Complex analysis for diagnosis of CCA in patients with PSC. Results from multi-biomarker analysis that reported on the diagnostic performance of these multivariant models are summarized separately for CCA on top PSC and CCA not associated with PSC.

Parameter	PSC vs. PSC-CCA				PSC vs. CCA				Ref.
	N (PSC/PSC-CCA)	AUROC	Sens. (%)	Spec. (%)	N (PSC/CCA)	AUROC	Sens. (%)	Spec. (%)	
Biliary proteomic analysis					18/25	0.87	78	84	[43]
Urinary proteomic analysis					45/42[a]	0.87	83	79	[44]
Combined biliary and urinary proteomic analysis					28/10[b]	0.84	94	76	[45]
Biliary lipidomic analysis					6/8	0.93	100	80	[47]
Biliary volatile organic compound analysis	21/11	0.89	91	73					[48]
Urinary volatile organic compound analysis					10/6	0.90	80	100	[49]
Biliary metabolomics analysis					5/5	n.r.	100	100	[50]

AUROC: area under the receiver operating characteristic curve; Sens.: sensitivity; Spec.: specificity; Ref.: reference; n.r.: not reported.
[a] Eighty-one patients with benign biliary disorder were included for calculation of these values; 10 patients with CCA had PSC-CCA.
[b] Twenty-nine patients with benign biliary disorder were included for calculation of these values, 6 patients with CCA had PSC-CCA.

Proteomics, lipidomics and metabolomics

Proteomics and lipidomics are the large-scale analysis of proteins and lipids and have gained much interest in PSC and CCA research in recent years. Especially with regard to potential CCA diagnosis such analyses have been performed in various body fluids.

The study by Lankisch et al. impressively showed the potential of bile proteomic analysis. In their study, the authors developed a model based on results of capillary electrophoresis mass spectrometry of bile fluid obtained during ERC to distinguish PSC from CCA with high accuracy. Their model yielded an AUC of 0.87 in ROC analysis, with specificity and sensitivity of 78% and 84%, respectively. Peptides identified and included in the model were mostly fragments of haemoglobin or cytoskeleton components, such as actin and keratin. As a drawback, of the 18 and 16 patients with PSC and CCA included in the training cohort and of the 18 and 25 patients with PSC and CCA in the validation set, only 10 patients with CCA developed CCA on top of PSC [43]. The same study group later reported on urine proteomic analysis, which has the great advantage of easier and safer accessibility compared with bile. The second study also resulted in a high AUC of 0.87 in ROC analysis for differentiation of CCA from PSC and other benign biliary disorders. Peptides identified mostly consisted of collagen fragments. As an advantage, that study included 10 patients with PSC-CCA that were all identified correctly [44]. A combined approach of biliary and urinary proteomic analyses was later performed and resulted in an AUC 0.84 for differentiation of CCA from benign biliary disorders, which was higher compared with biliary proteomic alone in the same study, but only a moderate improvement in AUC was found compared with urinary proteomic analysis alone. This study was also limited by including patients with PSC, CCA, PSC-CCA, and other benign biliary disorders [45]. Further evidence for the use of bile proteomic analysis was provided by Navaneethan et al. Their small study however only included 3 and 6 patients with CCA and PSC, besides patients with other pancreaticobiliary disorders. Despite the limitations in size, the study identified similar proteins to be more abundant in bile from patients with CCA compared with benign disorders as were identified by Lankisch et al., thus supporting their findings [46].

In another study by Navanetheen et al., lipidomic profiling with analysis of 10 different oxidized phospholipids in bile as markers of oxidative stress was performed. This study included patients with CCA ($n = 8$), PSC ($n = 6$), as well as pancreatic cancer and benign biliary disorders. Although the number of patients with CCA and PSC was low, and no information was provided on the presence of CCA on top of PSC, the presence of 1-palmitoyl-2-(9-oxononanoyl)-sn-glycero-3-phosphatidylcholine and 1-palmitoyl-2-succinoyl-sn-glycero-3-phosphatidylcholine well distinguished CCA from the other disorders, including pancreatic cancer. With regard to the small sample size and no information on the presence of PSC-CCA, an AUC of 0.93 for differentiation of CCA and PSC was reported [47]. Two further studies by the same authors analysed volatile organic compounds in bile and urine. Interestingly, the study analysing bile, included only patients with PSC (21 patients with PSC, and 11 with PSC-CCA). The study identified several differences in volatile organic compounds between both cohorts, and a logistic regression model including the substances acrylonitrile, 3-methylhexane, and benzene was developed. It yielded in an AUC of 0.89, with sensitivity and specificity of 91% and 73%, respectively. Volatile organic compounds were measured in the headspace of the bile by a selected ion flow tube mass spectrometry instrument. It would be of interest whether the same compounds are also exhaled and thus would be easily accessible for measurement [48]. The analysis of volatile organic compounds in urine was again limited by the heterogeneity of the study population, but

also showed a good diagnostic capability to distinguish CCA from benign biliary strictures [49].

Sharif et al. performed magnetic resonance spectroscopy in a metabolomic approach. Unfortunately, the study only included 5 patients with PSC and 5 with CCA and thus was limited by the very low study population of interest for this review. [50]. In a recent study, specific metabolic and signalling pathways were identified in a detailed analysis of patients with PSC [51]. All results are summarized in Table 4.

Considering the lack of validation studies and the complexity of the analyses, proteomics and lipidomics are not likely to become part of routine CCA screening in PSC patients in the foreseeable future. With regard to analyses from bile, the need for an invasive ERCP further limits their use. However, given the promising results, especially proteome analysis from urine and further analysis of volatile organic compounds, at best measured in exhaled air, should further be evaluated.

Conclusion

Several interesting and promising advances on biomarkers of PSC-associated CCA have been made, including improvements in the use of classical tumour markers, such as CA19-9, and novel approaches for the use of proteome analyses. However, despite the identification of several biomarkers in various body fluids, from a clinical perspective screening and diagnosing of CCA in PSC remains challenging. Most studies, despite showing promising results, were performed retrospectively in small cohorts often including patients with CCA without PSC and with other benign biliary disorders. In addition, results on most biomarkers are limited to single studies and confirmatory studies are rare. Although this limits the use of most biomarkers, other approaches, such as the proteomic or lipidomic analyses, are additionally limited by the complexity of the technique and are only available at specialized sites. Nevertheless, screening should be performed in PSC patients, as it might improve the outcome and survival of these patients [8].

To overcome most of the afore-mentioned limitations and to create a solid evidence, a prospective evaluation of the most promising biomarkers would be more than desirable. This study optimally would simultaneously investigate the most promising marker identified so far. Further research should as well focus on patients at increased risk of developing CCA, e.g., indicated by no reduction of ALP or by positive anti-GP2 IgA antibodies.

To summarize, the most promising results were found for serum CA19-9, particularly when combined with *FUT* genotyping, urinary proteomic analysis, and the evaluation of fucolin 2 and fibrinogen gamma chain in serum extra-cellular vesicles. Besides these, the analysis of volatile organic compounds seems interesting, because they might potentially be also detected during exhalation. Based upon the results of this review it however remains difficult to recommend a specific screening algorithm. Of all parameters CA19-9 has the great advantage of its wide availability and thus annual testing, even though there is no data to support an annual frequency, is recommended by some experts.

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AW and DG are co-owners of patents for a medical analysis systems that incorporate analysis of genotype for *fucosyltransferases* for colorectal and pancreatic cancer (DE102014109296B4/DE102014109296A1).

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