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BRAF V600E-specific immunohistochemistry reveals low mutation rates in biliary tract cancer and restriction to intrahepatic cholangiocarcinoma

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***BRAF* mutations have emerged as an important predictive biomarker for metastasized melanoma. Other types of cancer may also benefit from *BRAF* mutation-targeted therapies. In biliary tract cancer, reported *BRAF* mutation rates are highly controversial, ranging from 0 to 33% in adenocarcinoma of the gallbladder and 0 to 22% in cholangiocarcinoma. We here analyzed tissue microarrays of a large cohort of biliary tract cancer ($n=377$) including 159 intrahepatic cholangiocarcinomas, 149 extrahepatic cholangiocarcinomas, and 69 adenocarcinomas of the gallbladder for *BRAF* V600E mutation using a highly sensitive immunohistochemical screening approach implementing the BRAF V600E protein-specific antibody VE1. All VE1-positive cases as well as 42 VE1-negative cases were additionally analyzed by Sanger sequencing. In total, only 5 VE1-positive cases were detected (5/377; 1%). *BRAF* V600E mutation was confirmed by direct sequencing in all cases. All 5 mutated cases were intrahepatic cholangiocarcinomas (5/159; 3%). None of the extrahepatic cholangiocarcinomas and adenocarcinomas of the gallbladder were VE1 positive. Apart from the subtype restriction of *BRAF* V600E mutation to intrahepatic cholangiocarcinoma and a female predominance (4 female, 1 male), no significant correlation with clinicopathological data and patient outcome was detected. In conclusion, we demonstrate that *BRAF* V600E mutation is a rare event in biliary tract cancer, accounting for only 1% of all subtypes, and is restricted to intrahepatic cholangiocarcinoma. In addition, we demonstrate that VE1 immunohistochemistry is a feasible approach to routinely screen for *BRAF* V600E mutation in biliary tract cancer patients, thereby facilitating the detection of rare patients who may benefit from *BRAF* mutation-targeted therapies.**
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Biliary tract cancers are a diverse group of tumors that arise from the biliary tract epithelium. Biliary tract cancers can be divided into three major clinical phenotypes: cholangiocarcinomas of intrahepatic and extrahepatic origin and adenocarcinomas of the gallbladder. In the clinical context, biliary tract cancers are often treated as one disease, although there is evidence of genetic heterogeneity as well as differences in clinical behavior.[1] Most patients with biliary tract cancer present with unresectable or metastatic disease. Despite systemic chemotherapy, prognosis remains poor and to date there are no molecular-targeted therapies tailored to biliary tract cancer.[2]

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The RAS/RAF/MEK/ERK mitogen-activated protein (MAP) kinase pathway regulates cellular proliferation, survival, and migration, and is aberrantly activated in the majority of solid tumors. Mutation of the *BRAF* gene is one mechanism of constitutive activation and occurs in many human cancers including cutaneous melanoma, papillary thyroid carcinoma, borderline ovarian carcinoma, pleomorphic xanthoastrocytoma, colorectal carcinoma, non-small cell lung carcinoma, and hairy cell leukemia, among others.[3–6] The most common *BRAF* mutation results in a single amino-acid substitution of valine for glutamic acid at residue 600 (V600E). Early publications referred to codon 600 mutations as codon 599 mutations. In 2003, the *BRAF* sequence was updated with the insertion of 3 bp in the coding sequence, resulting in a new number of the hotspot codon (600 instead of 599). For metastatic melanoma, small-molecule inhibitors of BRAF V600E protein have demonstrated clinical activity and have rapidly changed standard treatment of *BRAF*-mutated melanoma patients.[7–9] Similarly, these targeted therapies might also have antitumor activity in various other *BRAF*-mutated neoplasias.[10–14] Accurate and rapid detection of *BRAF* mutations in metastatic melanoma is therefore essential for optimal patient care and may possibly be also required in other tumor entities in the near future.

An analysis of available data on *BRAF* V600E and non-V600E mutations revealed that *BRAF* mutation frequencies are highly controversial in biliary tract cancers ranging from 0 to 33% for *BRAF* V600E (see Table 1). As most studies with high *BRAF* mutation rates were performed on European cohorts, a recent review raised the question of whether these discordant results represent a regional difference in the genetics of biliary tract cancer.[2]

In this study, we investigated a large collection of biliary tract cancers for *BRAF* V600E mutations using a novel mutation-specific antibody (clone VE1) that specifically binds to BRAF V600E-mutated protein that is in general located in the cytoplasm. VE1 immunohistochemistry has proven to be highly sensitive and specific for various tumor types, including melanoma,[15–19] thyroid carcinoma,[20,21] colorectal carcinoma,[22,23] ganglioglioma,[24] and hairy cell leukemia,[25] among others.

The large numbers of the cohort investigated in this study should allow (1) to gain a more solid data basis for estimating the frequency of *BRAF* V600E mutation in biliary tract cancer, thereby partly resolving the discrepancy of the previously reported mutation rates, and (2) detecting associations of *BRAF* mutation status with subtypes and possibly other clinicopathological factors. Furthermore, we present a feasible approach to screen biliary tract cancer specimen for *BRAF* V600E mutation.

Materials and methods

Clinicopathological Characteristics of Biliary Tract Cancer Patients

Tissue samples from 377 patients (median age 64.1 years) who underwent bile duct and/or liver surgery at the University Hospital Heidelberg between 1995 and 2010 were included in this study. Only patients with primary adenocarcinomas of the biliary tract and without other known malignancies at the time of diagnosis were included. Biliary tract cancers consisted of 159 intrahepatic cholangiocarcinomas, 149 extrahepatic cholangiocarcinomas (106 perihilar and 43 distal), and 69 adenocarcinomas of the gallbladder. None of the patients received radio- and/or chemotherapy before surgery. Tumors were restaged according to the 7th TNM Classification of Malignant Tumors and classified after the World Health Organization (WHO) tumor classification system (WHO Classification of Tumours of the Digestive System, 4th edn, 2010) by two experienced pathologists (BG and WW). A summary of clinicopathological data is given in Table 2. The use of the tissues for this study was approved by the institutional ethics committee (206/05).

Tissue Microarray Construction

From all 377 biliary tract cancers, 3 μm sections were cut and stained with H&E. Representative areas

Table 1 Overview of studies that have examined *BRAF* mutation status in biliary tract cancer

Study	*Adenocarcinoma of the gallbladder BRAF V600E*	*Cholangiocarcinoma BRAF V600E*	*Intrahepatic cholangiocarcinoma BRAF V600E*	*Extrahepatic cholangiocarcinoma BRAF V600E*
Tannapfel *et al*[29]	Not investigated	11/69 (13%)[a]	CC subtype not specified	CC subtype not specified
Saetta *et al*[30]	7/21 (33%)	Not investigated	Not investigated	Not investigated
Goldenberg *et al*[31]	0/37 (0%)	0/25 (0%)	0/10 (0%)	0/15 (0%)
Borger *et al*[32]	0/25 (0%)	1/62 (2%)	1/40 (3%)	0/22 (0%)
Andersen *et al*[28]	Not investigated	1/69 (1%)	CC subtype not specified	CC subtype not specified
Sia *et al*[27]	Not investigated	3/141 (2%)[b]	3/141 (2%)[b]	Not investigated
Voss *et al*[26]	Not investigated	2/94 (2%)	2/67 (3%)	0/27 (0%)
This study	0/69 (0%)	5/308 (2%)	5/159 (3%)	0/149 (0%)

Abbreviation: CC, cholangiocarcinoma.
[a]Six additional non-V600E *BRAF* mutations detected (G469A, G469E, F595L, L597V, and 2 × V600D).
[b]Two additional non-V600E *BRAF* mutations detected (V600K and K601E).

were marked by two experienced pathologists (BG and WW). For each case, tumor tissue cores (1.0 mm diameter) from the selected representative tumor areas were punched out of the sample tissue blocks and embedded into a new paraffin array block using a tissue microarrayer (Beecher Instruments, Woodland, CA, USA).

Immunohistochemistry

Tissue microarray blocks were cut to 3 μm sections. BRAF V600E protein automated immunohistochemistry (clone VE1) was performed as previously described using the Ventana UltraView chemistry.[25] Scoring of immunostaining results was performed by two experienced observers (BG and DC) blinded to all clinicopathological and genetic data. Immunoreaction was scored positive, when viable tumor cells showed a nonambiguous cytoplasmic staining for VE1. A faint diffuse staining, any type of isolated nuclear staining, weak staining of single interspersed cells, or staining of monocytes/macrophages was not considered positive.

PCR Amplification and Direct Sequencing

In all selected cases (all immunopositive tumors and 42 randomly chosen immunohistochemistry-negative control intrahepatic cholangiocarcinoma cases), we performed direct sequencing of *BRAF* exon 15 as previously described.[3] In addition, we tested *BRAF* V600E-mutated cases for KRAS hotspot mutations by sequencing (complete exon 1) using standard primers. Microdissection was performed if tumor content was $<60\%$.

Statistical Analyses

Statistical analyses were performed with the statistical computing environment R version 2.15.1. Correlation analyses of *BRAF* V600E status with clinicopathological variables were assessed with Fisher's exact test. Univariate survival analysis was performed for overall survival by generation of Kaplan–Meier curves. Significance of differences between the groups was assessed using the log-rank test. The *P*-values of <0.05 were considered significant.

Results

Frequency of *BRAF* V600E Mutation in Biliary Tract Cancer and Correlation with Clinicopathological Data

Our immunohistochemical screen of 377 biliary tract cancers revealed 5 VE1-positive cases (1%). Immunoreaction was cytoplasmic, moderate to strong, and homogenous, staining all observable viable tumor cells in all five cases. Analysis of biliary tract

Table 2 Clinicopathological data of biliary tract cancer cohort with complete clinicopathological data and correlation with the *BRAF* V600E status

		Number (%)	
	Number (%)	*BRAF V600E*	*Fisher's exact test*
BTC patients	377 (100 %)	5 (1%)	NS
Age			
64–92 Years	187 (50%)	2 (1%)	NS
31–64 Years	190 (50%)	3 (2%)	NS
Sex			
M	190 (50%)	1 (1%)	0.37
W	187 (50%)	4 (2%)	NS
UICC (N = 296)			
UICC 1	40 (14%)	2 (5%)	NS
UICC 2	75 (25%)	1 (1%)	NS
UICC 3	82 (28%)	0 (0%)	NS
UICC 4	99 (33%)	2 (2%)	NS
pT			
T1	80 (21%)	1 (1%)	NS
T2	148 (39%)	3 (2%)	NS
T3	117 (31%)	1 (1%)	NS
T4	32 (9%)	0 (0%)	NS
pN (N = 286)			
N0	129 (45%)	2 (2%)	NS
N1	157 (55%)	2 (1%)	NS
M			
M0	354 (94%)	5 (1%)	NS
M1	23 (6%)	0 (0%)	NS
G			
G1	20 (5%)	0 (0%)	NS
G2	255 (68%)	4 (2%)	NS
G3	102 (27%)	1 (1%)	NS
L			
L0	174 (46%)	2 (1%)	NS
L1	203 (54%)	3 (2%)	NS
V			
V0	275 (73%)	3 (1%)	NS
V1	102 (27%)	2 (2%)	NS
Pn			
Pn0	294 (78%)	5 (2%)	NS
Pn1	83 (22%)	0 (0%)	NS
Biliary tract cancer subgroups			
Intrahepatic cholangiocarcinoma	159 (42%)	5 (3%)	0.01
Extrahepatic cholangiocarcinoma	149 (40%)	0 (0%)	NS
Adenocarcinomas of the gallbladder	69 (18%)	0 (0%)	NS
Histology			
Ductal	308 (82%)	4 (1%)	NS
Papillary	25 (7%)	1 (4%)	NS
Mucinous	10 (3%)	0 (0%)	NS
Intestinal	10 (3%)	0 (0%)	NS
Other	24 (6%)	0 (0%)	NS

cancer subtypes demonstrated that all 5 positive cases were intrahepatic cholangiocarcinomas (5/159, 3%), whereas all extrahepatic cholangiocarcinomas ($n=149$) and adenocarcinomas of the gallbladder ($n=69$) were VE1 negative (Figure 1 and Table 2). *BRAF* V600E mutation was confirmed by Sanger sequencing in all five cases. In 42 randomly chosen VE1 immunonegative intrahepatic cholangiocarcinoma control cases, no *BRAF* V600E and no other exon 15 hotspot mutation was detected by sequencing. Among the *BRAF*-mutated cases, no *KRAS* mutations were detected (data not shown). Despite the low number of detected mutations, the subtype restriction of *BRAF* V600E mutation to intrahepatic cholangiocarcinoma was statistically significant ($P=0.01$). A statistically nonsignificant female predominance (4 female, 1 male; $P=0.37$), but no further correlation of cases harboring *BRAF* V600E mutation with clinicopathological data or specific etiology (such as primary sclerosing cholangitis, HCV infection, Caroli disease), was detected (Table 2).

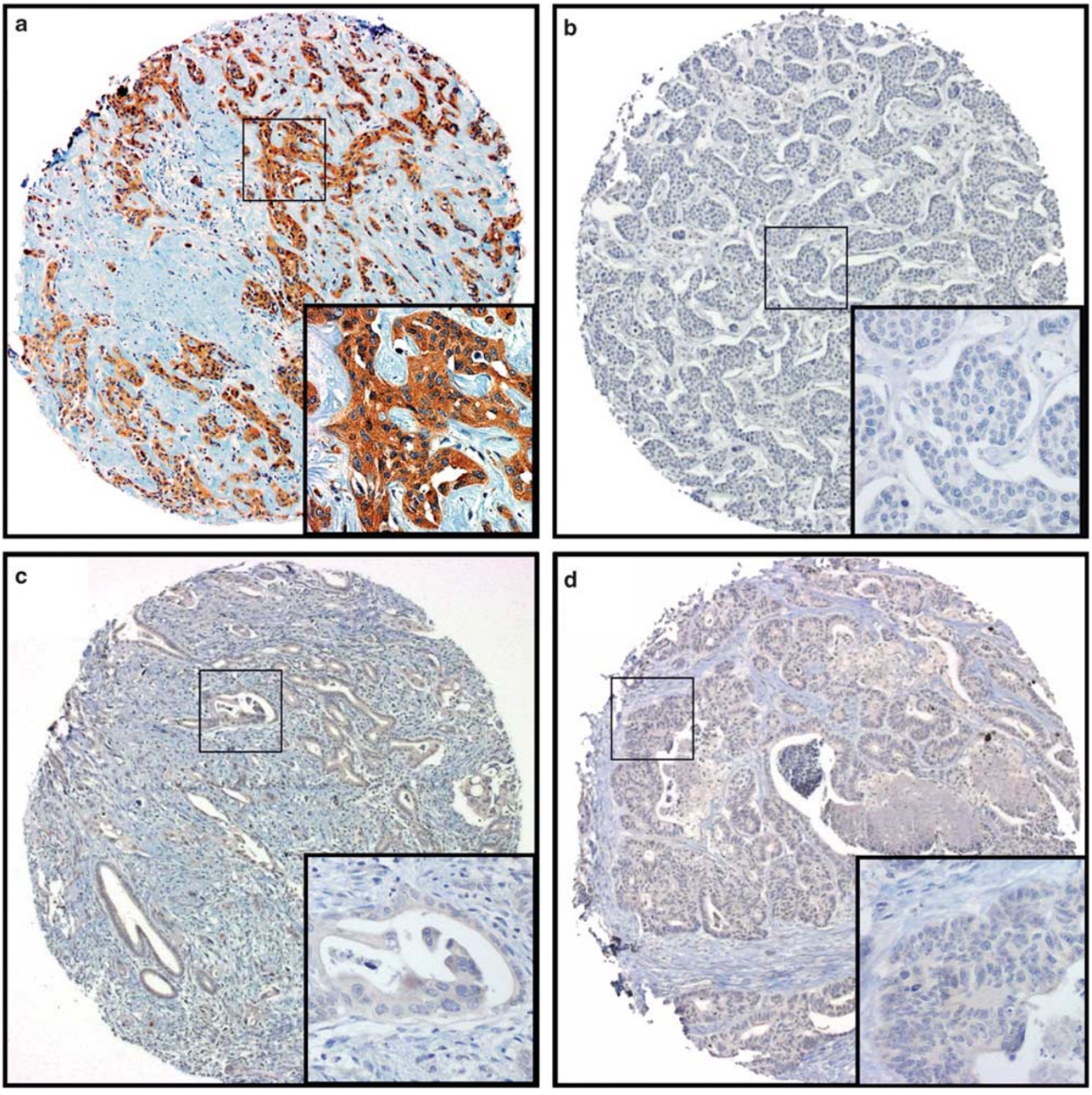

Figure 1 Representative micrographs of *BRAF* V600E-specific immunohistochemistry (clone VE1) of biliary tract cancer tissue microarray. (**a**) Strong homogenous cytoplasmic immunoreactivity in an intrahepatic cholangiocarcinoma. (**b**) Different intrahepatic cholangiocarcinoma with negative VE1 staining. (**c**) All extrahepatic cholangiocarcinomas ($n=149$) were negative for VE1. (**d**) All adenocarcinoma of the gallbladder ($n=69$) were negative for VE1. Magnification 50-fold, inset 200-fold.

Correlation of *BRAF* V600E Mutation Status with Survival of Intrahepatic Cholangiocarcinoma Patients

Among intrahepatic cholangiocarcinoma, patients with *BRAF* V600E-mutated tumors did not show an altered overall survival probability when compared with patients with *BRAF* wild-type tumors (Figure 2; $P = 0.38$).

Discussion

This analysis of a comprehensive biliary tract cancer cohort demonstrates that *BRAF* V600E mutation is a rare event in biliary tract cancer, accounting for only 1% of all cases ($n = 377$). Our findings are in line with recently published smaller series detecting *BRAF* mutations in 1% to 4% of evaluated cases.[26–28] We cannot confirm the exceedingly high mutation rates detected in early *BRAF* studies of European cohorts,[29,30] and conclude that the previously assumed regional difference in the genetics of biliary tract cancer in Europe *versus* the Americas is not existent.[2] Our data further demonstrate that *BRAF* mutations are restricted to intrahepatic cholangiocarcinomas and account for 3% in this subgroup. Comparing these results with earlier studies demonstrates that this restriction to intrahepatic cholangiocarcinoma is also evident in most preceding studies, although the case numbers were previously too low for further statistical conclusions (Table 1). A single early study reported several *BRAF* V600E-mutated cases among adenocarcinomas of the gallbladder.[30] Our data and the data of two other groups implementing diverse methods for mutation detection weaken the generalizability of this early observation in adenocarcinomas of the gallbladder.[31,32]

Most intrahepatic cholangiocarcinomas are diagnosed at an unresectable stage, for which only palliative chemotherapy remains as therapeutic option.[1,2] Thus, detection of a potentially treatable *BRAF* V600E mutation may represent an additional treatment option for these patients. The exclusive detection of *BRAF* V600E mutation in intrahepatic cholangiocarcinoma also has practical diagnostic implications, because the screening for *BRAF* V600E mutation in biliary tract cancer could be narrowed to this subtype, thereby substantially lowering the number of cases to be screened. Furthermore, our observation adds to the growing notion of a distinct molecular separation of intrahepatic and extrahepatic cholangiocarcinoma[1,2] as also recently observed for mutations of isocytrate dehydrogenase 1 and 2.[32]

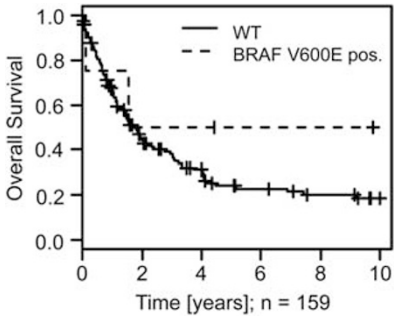

Figure 2 Overall survival probability in intrahepatic cholangiocarcinoma patients in correlation with *BRAF* V600E status. Kaplan–Meier curves show no difference in overall survival of patients in correlation with *BRAF* V600E status in intrahepatic cholangiocarcinoma ($P = 0.38$). The *P*-values were calculated with a log-rank test.

Correlation of *BRAF* V600E mutation status with survival of intrahepatic cholangiocarcinoma patients did not show any significant difference. However, this conclusion is of limited validity because of the low number of observed mutated cases. In other types of cancer, *BRAF* mutations have shown varying association with patient mortality, but the effect is generally not independent of other tumor features, and thus *BRAF* status currently does not give definite prognostic implications for most tumor types.[33,34]

Implementation of VE1 immunohistochemistry for the prediction of a *BRAF* V600E mutation has been investigated by various groups and for various types of cancer.[15–21,23–25] VE1 immunohistochemistry has the advantage of being rapid and relatively inexpensive, while the sensitivity and specificity are comparable to DNA-based methods. For pathologists, an additional well-appreciated advantage is the *in situ* mutation detection that provides information on the mutated cell population, as well as an additional visual sample verification that a faceless DNA sample does not offer. The utilization of such a technique seems particularly feasible when the expected rate of mutations is as low as in intrahepatic cholangiocarcinoma, and routine implementation of more work-intense methods such as direct sequencing is less attractive. Use of VE1 to screen for low-frequency *BRAF* V600E mutations has previously been demonstrated for primary lung adenocarcinoma and for unselected brain metastases of various histologies.[35,36]

A clear disadvantage is that the immunohistochemical mutation analysis is limited to *BRAF* V600E. This is of highest relevance in melanoma, where depending on tumor genesis (especially sun exposure) up to 25% of *BRAF* mutations may be non-V600E.[34] In biliary tract cancer, the vast majority of *BRAF* mutations reported to date were of the V600E type, with the limitation that several studies used DNA-based methods that are only able to detect a limited spectrum of *BRAF* mutations (eg, the Prism SNaPshot Multiplex system that can detect V600A, E, G, and M[32]). In two previously analyzed cholangiocarcinoma cohorts, tumors with *BRAF* mutations other than V600E have been reported that may potentially also respond to targeted therapy. For *BRAF* V600K (detected in one intrahepatic cholangiocarcinoma by Sia *et al*[27]) response of melanoma metastases has been observed in a phase II trial,[37] and for *BRAF* V600D (detected in two cholangiocarcinomas not otherwise specified by Tannapfel *et al*[29]) *in vitro* inhibitor

activity was shown in a melanoma cell line.[38] In single instances, other rare *BRAF* mutations were detected (eg, *BRAF* K601E) for which data on potential clinical activity of *BRAF* inhibitors are not available. VE1 immunohistochemistry does not detect V600K, V600D, or other rare mutations,[15,16] and would thus fail to identify these potential candidates for targeted therapy. In our collection of 42 VE1-negative intrahepatic cholangiocarcinomas, we did not observe any non-V600E mutations, indicating that they altogether likely represent rare events in intrahepatic cholangiocarcinoma.

In conclusion, we demonstrate that VE1 immunohistochemistry is a feasible and valid approach for screening for *BRAF* V600E mutation in biliary tract cancer. In biliary tract cancer, *BRAF* V600E mutation is restricted to intrahepatic cholangiocarcinomas, and screening of extrahepatic cholangiocarcinomas and adenocarcinomas of the gallbladder could therefore likely be omitted. Considering that ~70 000 new cases of intrahepatic cholangiocarcinoma are diagnosed globally every year, ~2000 to 3000 patients are expected to harbor *BRAF* V600E-mutated tumors.[27] These patients may benefit from targeted therapies, and *BRAF* mutation screening approaches—like the one presented in this study—should be evaluated for design of prospective trials for such low *BRAF* mutation frequency populations.

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Disclosure/conflict of interest

Under a licensing agreement between Ventana Medical Systems, Tucson, Arizona, and the German Cancer Research Center, DC and AvD are entitled to a share of royalties received by the German Cancer Research Center on the sales of VE1 antibody. The terms of this arrangement are being managed by the German Cancer Research Center in accordance with its conflict of interest policies.

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