Heterozygosity for the alpha1-antitrypsin Z allele may confer genetic risk of cholangiocarcinoma

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Heterozygosity for the *alpha1-antitrypsin* Z allele may confer genetic risk of cholangiocarcinoma

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Mihalache et al. Cholangiocarcinoma risk in $\alpha 1AT$ Z heterozygosity

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ABSTRACT

Background & Aim: Alpha1-antitrypsin ($\alpha 1AT$) deficiency caused by Z allele homozygosity represents a risk factor for hepatocellular carcinoma. Previous studies have implicated $\alpha 1AT$ Z heterozygosity in cholangiocarcinogenesis. We assessed the “common” Z and S alleles as well as the promoter variant *rs8004738* for association with cholangiocarcinoma (CCA).

Patients & Methods: We genotyped 182 Caucasian patients and 350 controls for *rs28929474* (Z), *rs17580* (S) and the variant *rs8004738*. Exploratory analyses were performed in relation to gender and CCA localization.

Results: *rs28929474* was significantly enriched in the CCA group (4.1 vs. 1.7 %; OR 2.46, 95% CI 1.14-5.32: Bonferroni corrected $p_c=0.036$), reinforced by Armitage trend testing (OR 2.53: $p_c=0.032$). *rs8004738* (promoter) minor allele tended to be overrepresented in Z heterozygotes (30.0 vs. 16.7 %: $p=0.13$). Exploratory data analyses suggested a high genetic risk for extrahepatic localization (OR 3.0: $p_c=0.016$) and potentially female Z allele carriers (OR 3.37: unadjusted $p=0.022$, $p_c=0.088$).

Conclusion: These data point to a novel role of $\alpha 1AT$ Z heterozygosity as a potential genetic susceptibility factor for CCA formation, and may suggest a contribution of aberrant $\alpha 1AT$ function in biliary carcinogenesis. However, given the overall low *rs28929474* minor allele frequency, larger studies are warranted to confirm and extend our findings.

INTRODUCTION

Deficiency for alpha1-antitrypsin ($\alpha 1AT$) encoded by the polymorphic *SERPINA1* gene (alternatively referred to as *protease inhibitor PI*) is, in its traditional form, caused by homozygosity for mutant Z alleles. Unlike $\alpha 1AT$ lung disease related to blunted protease inhibitor function, the liver phenotype results from protein misfolding and defective export from hepatocytes, the major cellular source of circulating $\alpha 1AT$, causing retention and polymerization in the endoplasmic reticulum (ER), reminiscent of a “gain-of-toxic function” mechanism.[1] Conversely, the variant S allele undergoes premature intracellular degradation and evokes subtle reductions in serum levels without causing classic clinical liver disease, unless a Z allele (or other rare polymerization-prone $\alpha 1AT$ variants) is co-inherited.[2] Likewise, the clinical penetrance of $\alpha 1AT$ liver disease, representing the most common hereditary liver disease in childhood, has been reported to undergo intragenic modulation by the common $\alpha 1AT$ promoter variant *rs8004738*. [3] Moreover, $\alpha 1AT$ deficiency constitutes a well-recognized risk factor for hepatocellular carcinoma (HCC) with or without established cirrhosis.[4-5] However, pathology-based studies assessing tumoral and non-tumoral liver samples for immunohistochemical PiZ deposits in Z allele heterozygotes revealed an unexpectedly high rate of primary cholangiocarcinoma or combined liver tumors with marked cholangiocellular differentiation.[6-7]

Cholangiocarcinoma (CCA) is a devastating cancer arising from the epithelial lining of intra- or extrahepatic bile ducts with rising incidence rates worldwide.[8] Though its molecular pathogenesis remains ill defined, the combined effects of chronic cholestasis and inflammation are considered key to the malignant transformation of cholangiocytes. Evidence for potential

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3 risk genes relevant to cholangiocarcinogenesis is scarce, in part due to its overall low incidence
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6 rate and the ensuing difficulties in establishing study cohorts of adequate sample sizes.
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9 Notwithstanding, a better characterization of genetic susceptibility factors may help pinpoint
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11 relevant carcinogenic pathways and could impact on clinical management in the future.[9]
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14 From this perspective, our aim was to assess the putative association of the “common”
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16 $\alpha 1AT$ -deficient alleles Z and S with CCA susceptibility in a large European-based cohort. As
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18 secondary study hypotheses, the potential risk modulation by *rs8004738* as a *bona fide*
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20 modifier in $\alpha 1AT$ deficiency phenotypes and subgroup analyses in relation to either extra- or
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22 intrahepatic tumor localization and potential gender disparities had to be addressed.
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PATIENTS & METHODS

Study Design and Population

We recruited a total of 182 Caucasian CCA patients and 350 controls from three academic medical centres in Germany (Bonn and Homburg: 152/290) and Romania (Cluj Napoca: 30/60); 128 cases were prospectively enrolled and an additional 54 patients with available archived blood samples were retrospectively included (Department of Virology, Homburg). CCA diagnosis was confirmed by compatible histology including specialized immunohistochemistry in 71.5 % and cytology in 12.0 % in conjunction with appropriate exclusion of extrabiliary malignancy. Bile duct carcinomas were classified as either intra- or extrahepatic CCAs as recommended by the British Society of Gastroenterology.[10] Alternatively, the diagnosis was based on clinical grounds in 16.5 % taking into account detailed cross-sectional and, partly advanced, biliary imaging studies, follow-up data, and exclusion of potential alternative diagnosis as per review of medical charts. Controls were recruited on the occasion of colorectal cancer screening (290 individuals all with negative colonoscopy and negative cancer history) and/or abdominal ultrasound for exclusion of gall stone disease (60 individuals all without evidence for cholelithiasis). All participants gave written informed consent, and the study was approved by the local ethic committees and in accordance with the revised Declaration of Helsinki.

Genotyping

Genomic DNA was isolated using the QIAamp protocol (Qiagen, Hilden, Germany). DNA concentration was calibrated to 5-50 ng/ μ l with the NanoDrop ND-1000 spectrophotometer

(NanoDrop Technologies, DE, USA). Genotyping for *rs17580* (S allele), *rs28929474* (Z allele) and *rs8004738* was performed using solution-phase hybridization reactions with 5'-nuclease and fluorescence detection (TaqMan assays, Applied Biosystems, Foster City, CA, USA) on an ABI 7500 Fast Real-Time polymerase chain reaction (PCR) system (Applied Biosystems, Norwalk, CT, USA). PCR reactions contained 2 x Genotyping Mastermix 5 μ l, 40 x Genotyping Mix 0.25 μ l, H₂O 3.75 μ l, DNA 5-50 ng/ μ l. Amplification condition were as follows: 95°C for 10 min, 40 cycles of 95°C for 10 s and 60°C for 1 min.

For comparison and validation, selected samples were genotyped by a different methodology (*DNA-STRIP® technology*) involving generation of a single-stranded amplicon, probe-specific hybridization and alkaline phosphatase-based detection on a membrane strip (GenoType® AAT kit, Hain Lifescience GmbH, Nehren, Germany).

Statistical analysis

Data are given as median and ranges, and p values <0.05 are considered significant. Age and gender distributions in cases and controls were compared using t-test and univariate logistic regression analysis, respectively.

Genotype distributions for each SNP were tested for consistency with Hardy-Weinberg equilibrium (HWE) in controls (p>0.05) using an exact test and visualized in triangular de Finetti diagrams (<http://ihg.gsf.de/snps.html>). Allele and genotype frequencies were compared using χ^2 tests, and Armitage trend tests were used to compare genotype distributions between cases and controls in 2 x 3 tables.

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3 Bonferroni corrections were applied as appropriate (correction by factor 2 in the
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5 primary study approach evaluating for Z and S alleles; multiple testing in the secondary study
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7 hypothesis as for gender and CCA localization corrected for by factor 4) to reduce the potential
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9 of type I errors. Bonferroni adjusted p values are indicated as p_c .
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13 Statistical analysis was performed with the SPSS software package (version 16.0: SPSS,
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15 Munich, Germany). Power calculations were performed by using *PS: Power and Sample Size*
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17 *Calculation v.3.0* (<http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize>).
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RESULTS

As for gender distribution, 57 % of subjects in the CCA group were male and 43 % female, whereas in the control group 43 % were male and 57 % female ($p=0.003$). The median age at study entry/at diagnosis was significantly higher in cases (67.3 ± 11.8 vs. 61.3 ± 11.4 ; $p=0.0001$). There was a marked dominance of tumors located in the extrahepatic biliary system (141 extra- vs. 41 intrahepatic CCAs). **Table 1** summarizes the demographic and clinical parameters of cases and controls. Given the significant differences in age and gender structure, we performed uni- and multivariate logistic regression analysis with a stepwise forward approach indicating that any factor analyzed (gender, age and $\alpha 1AT$ Z genotype) was significantly but independently associated with CCA risk in our dataset consisting of age- and gender-unmatched controls. (**Table 2**) The results of the power analysis are given in **Figure 1**.

Genotyping for *rs28929474* (Z) was successful in all subjects, while allelic discrimination failed to unequivocally assign an *rs17580* (S) genotype in 1/532 and for *rs8004738* (promoter) in 3/532 individuals. Collectively, call rates > 99.99 % were determined for any SNP. We re-genotyped > 10 % of the sample set and all Z allele carriers yielding near-completely congruent results except for one Z allele carrier with an ambiguous second allelic discrimination read-out. In this individual, Z allele heterozygosity was confirmed by applying a different genotyping platform according to the manufacturer’s protocol (see *Methods*).

Allele frequencies were compatible with *Entrez SNP database* entries (<http://www.ncbi.nlm.nih.gov/snp>), and no deviation from Hardy-Weinberg equilibrium was detected ($p>0.05$ for any SNP). We identified a total of 27 Z allele heterozygote individuals (15 cases vs. 12 controls) and likewise 27 S allele heterozygotes (9 cases and 18 controls). There

was one single SZ individual (66-year-old man with extrahepatic CCA), while no homozygotes for either Z or S were identified. No significant differences in allele and genotype distributions were observed for *rs17580* (S). However, *rs28929474* (Z) was significantly enriched in the CCA group (4.1 vs. 1.7 %; OR 2.46, 95% CI 1.14-5.32; $p_c=0.036$), and Armitage trend testing reinforced the association of *rs28929474* genotypes with CCA risk (OR 2.53; $p_c=0.032$) (**Table 3**).

As regards the $\alpha 1AT$ promoter single nucleotide polymorphism (SNP), we only considered Z heterozygotes in the CCA and control group (mean age 65.5 ± 11.4 vs. 59.9 ± 11.4 : not significant (ns); males/females 6/9 vs. 5/7: ns). A strong, though formally non-significant trend towards an overrepresentation of the *rs8004738* minor allele in the Z heterozygous CCA group was detected (9/30 vs. 4/24 or 30.0 vs. 16.7 %; OR 2.6, $p=0.14$) (**Table 4**).

Moreover, subgroup analyses stratifying in relation to gender and tumor localization indicated a particular high genetic risk for extrahepatic tumors (OR 3.0, 95% CI 1.37-6.56; $p_c=0.016$) and in females (OR 3.37, 95% CI 1.23-9.20: unadjusted $p=0.022$) in Z allele carriers. However, this gender-specific association fell short of passing Bonferroni corrected significance levels ($p_c=0.088$) (**Table 5**).

DISCUSSION

The low incidence and prevalence of CCA complicates the establishment of informative study cohorts, and may thus contribute to the relative paucity of suspected or appreciated genetic risk factors underlying biliary carcinogenesis.[9, 11] The current collaborative study in one of the largest, European-based CCA cohorts, including 182 patients, represents the first genetic-based study investigating the contribution of α 1AT-deficient alleles to CCA risk. We provide preliminary evidence for a genetic risk modulation by the Z allele with fairly considerable effect sizes on biliary cancer risk (OR 2.46, $p_c=0.036$). Of note, the overall association was substantiated by positive Armitage trend testing and passed Bonferroni corrections as the most conservative statistical approach. Given the generally low Z allele frequency (1.7 vs. 4.1 % in controls vs. cancer patients), compatible with a rare disease variant potentially accounting for pronounced genetic risk effects, the population attributable risk (PAR) of the identified genetic contribution may be viewed as of limited clinical relevance.[12] However, the assumed relevance of the suggested genetic association may rather to be seen in the potential implication of aberrant α 1AT function in biliary tract carcinogenesis.

Not entirely unexpected extrapolating available data from classic α 1AT liver disease, the S allele *per se* did not associate with CCA susceptibility in our analysis. In light of just one SZ carrier (interestingly, in the CCA cohort), a reasonable evaluation of the assumption that the S allele might rather act as a potential modifier than as causal variant was precluded. In the same direction, the promoter variant tended to be enriched in the CCA group, though this did not reach statistical significance either. Nevertheless, a modifier effect by *rs8004738* on α 1AT-

related cholangiocarcinogenesis may not be firmly dismissed, given the low rate of Z allele positivity and, thus, low statistical power as to such dedicated analysis.

Of note, in our **hypothesis generating exploratory data** analyses, extrahepatic cancer sites and female gender were **potentially** associated with CCA risk. In the study by Zhou et al.[6] heterozygosity for the Z allele has been linked to intrahepatic CCA, **presumably** arising from bipotent periportal progenitor cells considered to represent a nidus for biphasic primary liver cancers and/or subsets of intrahepatic CCA.[13] In our dataset, we were not able to replicate such a finding, although given the confined number of intrahepatic CCAs, comprising no more than 41 cases, our study is certainly underpowered to be confident in refuting this specific association (potential type II error). Confirmatory pathologic research establishing the distinct cellular origin of $\alpha 1AT$ -related intrahepatic CCAs and genetic studies in larger cohorts are warranted. The gender disparity unfavoring women in our study is unexpected in that previous epidemiologic and PiZ transgenic mouse model data suggest that $\alpha 1AT$ -associated hepatocarcinogenesis appears to be confined to males. [5, 14] However, it is of interest to note that recent experimental work provides ancillary evidence for estrogen-dependent gender differences in malignant and non-malignant biliary epithelial cell (BEC) IL-6 signalling, considered to represent one major inflammatory driver in cholangiocarcinogenesis.[15]

The **presumed** overrepresentation of Z alleles in extrahepatic CCAs, **to a large extent** accounting for the detected global association merits discussion as well, since this **might represent** an altogether novel observation. We can only speculate as to how Z heterozygosity may be implicated in the pathogenesis of extrahepatic CCAs. First, in situ production of $\alpha 1AT$ at low levels might occur in large-duct cholangiocytes. Following **this hypothesis**, retention and

polymerization of the misfolded Z protein might ensue, as has been demonstrated for bronchial epithelial cells.[16-17] However, while there is scant evidence for minor $\alpha 1AT$ expression in intrahepatic bile ducts, no sustainable data are available with respect to the larger, extrahepatic bile ducts, where the relative cancer risk appeared to be the most prominent in the current study.[18-19] Alternatively, conceivable alterations in bile compositions by the Z allele might, on a speculative basis, promote inflammation-driven biliary carcinogenesis. Indeed, provisional data as to biliary $\alpha 1AT$ functions indicate that it is present in bile at low levels, however, mostly non-functional.[20-21] Taken together, the potential role of $\alpha 1AT$ in biliary physiology and pathophysiology remains yet to be defined.

Several limitations of our study have to be critically addressed in particular with respect to the limited statistical power for a dedicated subgroup analyses and to the basic study design with unbalanced age and gender structures between cases and control groups. Notwithstanding, we provisionally conclude that the presented data may provide a first evidence for an increased genetic CCA risk in Z allele carriers potentially of particular relevance for extrahepatic CCA formation and female gender. However, these novel findings clearly have to be replicated and extended in larger and independent cohorts. In view of the lack of knowledge as to the potential carcinogenic roles of biliary $\alpha 1AT$ expression and/or secretion, the potential molecular mechanisms by which $\alpha 1AT$ might be involved in CCA pathogenesis would have to be elucidated.

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Statement of Interests

Nothing to declare.

For Peer Review

TABLES

	CCA (n=182)	Controls (n=350)	p value
Age, years (mean $\pm$ SD)	65.8 $\pm$ 11.35	61.0 $\pm$ 11.1	0.003
Gender, male/female (%)	103/79 (57/43)	151/199 (43/57)	0.0001
Localization (intra-/extrahepatic) (%)	41/141 (22/78)		
Tissue diagnosis (%)	152 (84)		
Clinical diagnosis (%)	30 (16)		

Table 1: Demographic and clinical parameters of study subjects**(A) Univariate analysis**

Factor	OR	95% CI	p value
<i>rs28929474</i> (Z)	2.53	1.16 – 5.53	0.020
Age	1.04	1.02 – 1.06	< 0.001
Gender (male)	1.72	1.20 – 2.47	0.003

(B) Multivariate analysis

Factor	OR	95% CI	p value
<i>rs28929474</i> (Z)	2.81	1.26 – 6.31	0.012
Age	1.04	1.03 – 1.06	< 0.001
Gender (male)	2.04	1.39 – 2.98	< 0.001

Table 2: Uni- and multivariate logistic regression analysis using a stepwise forward approach. (OR odds ratio, CI confidence interval)

	CCA (n=364)	Controls (n=700)	p value (p_c)
rs28929474 - Z (%)			0.018 (0.036)
C	349 (96)	688 (98)	(OR 2.46)
T	15 (4)	12 (2)	[95% CI 1.14-5.32]
rs17580 - S (%)			*
T	355 (98)	680 (97)	(OR 1.04)
A	9 (2)	18 (3)	[95% CI 0.46-2.34]

Table 3: Allele frequencies of rs28929474 (Z) and rs17580 (S) (* one undetermined subject; p_c Bonferroni corrected p value)

	CCA (n=15)	Controls (n=12)	p value
Age, years (mean $\pm$ SD)			
	65.5 $\pm$ 11.5	59.9 $\pm$ 11.4	ns
Gender, m/f (%)			
	6/9 (40/60)	5/7 (42/58)	ns
rs8004738 alleles (%)			ns (0.14)
T	21 (70)	20 (83)	(OR 2.62)
C	9 (30)	4 (17)	[95 %CI 0.72-9.59]
rs8004738 genotypes (%)			
TT	7 (47)	8 (67)	ns (0.14)*
TC	7 (47)	4 (33)	OR 6.07
CC	1 (7)	0 (0)	

Table 4: Demographic and rs8004738 (promoter) genotyping data in Z allele carriers in the study population (* Armitage trend test)

	CCA	Controls	p value (p_c)
Gender (%)			
Males	200/6 (97.1/2.9)	297/5 (98.3/1.7)	ns
Females	149/9 (94.3/5.7)	391/7 (98.2/1.8)	0.022 (0.088)
Localization (%)			
Intrahepatic	81/1 (98.8/1.2)	688/12 (98.3/1.7)	ns
Extrahepatic	288/14 (95.4/4.6)		0.004 (0.016)

Table 5: Allele distribution of *rs28929474* (Z) after stratification for gender and tumor localization (p_c Bonferroni corrected p value)

FIGURE LEGEND

Figure 1: Power analysis using *PS: Power and Sample Size Calculation v.3.0*: Statistical power ($\alpha=0.05$) as a function of the effect size (odds ratio) and minor allele frequencies (MAF) in the control group.