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ORIGINAL ARTICLE

Mutation analysis of the *PALB2* gene in unselected pancreatic cancer patients in the Czech Republic

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Pancreatic ductal adenocarcinoma (PDAC) has the worst prognosis among common solid cancer diagnoses. It has been shown that up to 10% of PDAC cases have a familial component. Characterization of PDAC-susceptibility genes could reveal high-risk individuals and patients that may benefit from tailored therapy. Hereditary mutations in *PALB2* (Partner and Localizer of BRCA2) gene has been shown to predispose, namely to PDAC and breast cancers; however, frequencies of mutations vary among distinct geographical populations.

Using the combination of sequencing, high-resolution melting and multiplex ligation-dependent probe amplification analyses, we screened the entire *PALB2* gene in 152 unselected Czech PDAC patients. Truncating mutations were identified in three (2.0%) patients. Genotyping of found *PALB2* variants in 1226 control samples revealed one carrier of *PALB2* truncating variant (0.08%; P = 0.005). The mean age at PDAC diagnosis was significantly lower among *PALB2* mutation carriers (51 years) than in non-carriers (63 years; P = 0.016). Only one patient carrying germline *PALB2* mutation had a positive family breast cancer history.

Our results indicate that hereditary *PALB2* mutation represents clinically considerable genetic factor increasing PDAC susceptibility in our population and that analysis of *PALB2* should be considered not only in PDAC patients with familial history of breast or pancreatic cancers but also in younger PDAC patients without family cancer history.

Keywords Pancreatic ductal adenocarcinoma, *PALB2* gene, hereditary mutations

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most aggressive and lethal diagnosis among all solid malignancies with only about 7% of patients surviving five years from the diagnosis (1). The median age at diagnosis is 72 years and development of PDAC before the age of 50 is rare (2). PDAC is the fifth most frequent cause of cancer death in the Czech Republic (www.svod.cz) and its high incidence brought Czech Republic to the first place in PDAC incidence worldwide (3).

Surgical resection combined with chemotherapy is currently the only treatment for PDAC improving the survival; however, treatment is often limited to the chemotherapy with very low efficacy due to the late diagnosis in majority of patients. Causes of PDAC are largely unknown although several environmental, behavioral and genetic risk factors have been described including age, sex, race, cigarette smoking, diets high in meats and fat, low serum folate levels, obesity, long-standing diabetes mellitus and chronic pancreatitis (2,4). Numerous studies have shown that approximately 10% of PDAC patients have a first degree relative with PDAC, indicating an important contribution of genetic factors to PDAC development (2). Several PDAC-susceptibility genes increasing the risk of tumor development in carriers of their mutations have been described (reviewed in Reference (5)). Among them, *BRCA2* has been considered as the major susceptibility gene that accounts for

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the development of 6% of all familial cases (6) increasing the PDAC risk 3.5–10 fold (7). Apart from *BRCA2*, an important role is also attributed to the *PALB2* (Partner and Localizer of BRCA2) gene that codes for a protein participating in the DNA double-strand break repair in complex with BRCA2. Pathogenic hereditary *PALB2* mutations were associated with increased risk of several cancer types including breast, male breast, ovarian, and pancreatic ductal adenocarcinoma, and less frequently with prostate, colorectal and melanoma cancer (8–11). Bi-allelic mutations in *PALB2* cause rare autosomal recessive cancer-prone syndrome Fanconi anaemia subtype N (FANCN) (12). The majority of studies focused on characterization of hereditary *PALB2* mutations in high-risk breast cancer patients; however, the growing evidence indicates that hereditary *PALB2* mutations represent also an important genetic factor contributing to the pancreatic cancer development.

During our previous analyses we have shown that high-risk breast cancer patients from hereditary breast cancer families in the Czech Republic have high incidence of *PALB2* variants (13). In this study, we aimed to determine the prevalence and spectrum of hereditary *PALB2* mutations among unselected PDAC patients in the Czech Republic.

Materials and methods

Patients

We have analyzed the entire coding sequence of the *PALB2* gene in 152 blood-isolated DNA samples obtained from individuals with histopathologically-verified PDAC diagnosed between the years of 2004 and 2012. The samples were collected at the National Institute of Public Health in Prague (14). Median age at diagnosis of all PDAC patients was 63 years ranging from 40 to 82 years. Patients, 59 females and 93 males, were not selected for age, gender, or family history. Control group of 1226 individuals was analyzed previously in Janatova et al. (13). All patients and controls were Caucasians of the Czech origin from the Prague area. The study was approved by the local Ethical Committees and a written informed consent with the use of archived DNA samples for research purposes was obtained from all participants.

Mutation analysis

We analyzed all 13 *PALB2* coding exons and flanking intron-exon boundaries. Exons 4, 5, and 6, comprising 67% of the *PALB2* genomic sequence were directly amplified and sequenced. PCR reaction consisted of 1.5 μl of 10× PCR buffer, 0.2 mM of each dNTP, 0.4 μM of each primer, 10 ng of DNA template, and 0.5 U of Fast Start Taq DNA polymerase (Roche) in reaction volume of 15 μl. PCR reactions were incubated at 35 cycles (95 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 2 minutes) with a final extension at 72 °C for 10 minutes. All primer sequences are listed in Supplementary Table S1. Amplified fragments were purified by ExoSAP-IT (USB Corp.) and sequenced using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and analyzed by ABI 3130 (Applied Biosystems) according to manufacturer's instructions.

Remaining short exons were screened using High Resolution Melting Analysis (HRMA) with HOT Fire Pol EvaGreen HRM Mix (Solis BioDyne) and the analysis of melting curves was performed on the LightCycler 480 (Roche) according to the manufacturer's instructions. Melting profiles were evaluated at LightCycler 480 Software ver. 1.5.0 SP4 (Roche) and potential mutations were confirmed by sequencing from independent PCR reactions as mentioned earlier.

Presence of large genomic rearrangements (LGRs) was analyzed by the Multiplex Ligation-dependent Probe Amplification (probe mix P260-A1; MRC-Holland) as described previously (13).

Identified *PALB2* mutations were genotyped in controls by HRMA as described previously (13).

In silico analysis

Pathogenicity of missense variants was assessed by prediction tools: PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/), Align-GVGD (http://agvgd.iarc.fr/), Mutation taster (http://www.mutationtaster.org/), and CADD score (http://cadd.gs.washington.edu/). Their frequency was compared to available databases: ESP (http://evs.gs.washington.edu/EVS/), 1000 Genomes (http://www.1000genomes.org/), and ExAC (http://exac.broadinstitute.org/).

Statistical analysis

Difference between groups was calculated using Fisher's Exact Test and Mann–Whitney Test.

Results

In our group of 152 unselected Czech PDAC patients, we found three *PALB2* pathogenic truncating mutations (3/152; 1.97%; Table 1). Two of them were already described (c.509_510delGA

Table 1 List of truncating *PALB2* variants found in 152 unselected Czech patients with pancreatic ductal adenocarcinoma

Nucleotide change	Protein change	Exon[a]	Diagnosis (age); gender	Family cancer history (age at diagnosis)[c]	References
c.509_510delGA[b]	p.Arg170fs	4	48; male	0	(13,15–19)
c.697delG	p.Val233fs	4	50; male	M—unknown tumor (50) F—gastric (74)	(20,21)
c.1838delA	p.Gln613fs	5	56; male	M—breast (53)	Novel

Abbreviations: M, mother; F, father.

[a] NCBI reference sequence: NG_007406.

[b] This variant was the only truncating variant identified in 1226 controls.

[c] DNA samples from relatives with cancer diagnoses were unavailable for segregation analyses.

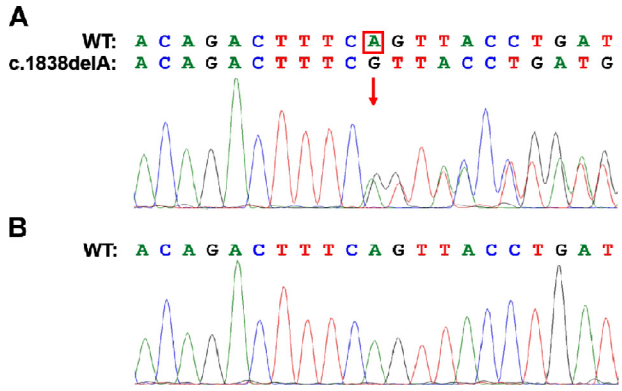

Figure 1 Sequencing of the novel hereditary *PALB2* mutation c.1838delA (deleted nucleotide indicated in the red box) in heterozygous mutation carriers (A) shown with corresponding WT DNA sequence (B).

and c.697delG), the third was novel (c.1838delA; Figure 1). The MLPA analysis revealed no LGR in analyzed patients. Only one mutation (c.509_510delGA) was found in control samples (1/1226; 0.08%; P = 0.005). We found no PDAC in family cancer history of patients carrying truncating *PALB2* mutations (Table 1). The mean age at diagnosis of *PALB2* mutations carriers (51 years) was significantly lower than that in non-carriers (63 years; P = 0.016).

We found another 13 missense and silent variants alongside to the truncating mutations (Supplementary Table S2). Among them, c.3494C>T (p.Ser1165Leu) was a rare missense variant, considered as a pathogenic by all prediction softwares (except to AlignGVGD), and was the only SNV not listed in the NCBI SNP database. The male carrier of this variant was diagnosed with PDAC at 50 years with no family cancer history.

Discussion

The *PALB2* gene was identified as a pancreatic cancer-susceptibility gene by Jones et al. (22,23) who perform a comprehensive genetic analysis of 24 PDAC patients including the next-generation sequencing (NGS) analysis. An extension of this initial study found 3 carriers of truncating *PALB2* mutation among 96 PDAC patients (3.1%) from families, where PDAC affects two or more first degree relatives (23). Later studies have shown slightly higher frequency of *PALB2* mutation carriers in patients from families with pancreatic or breast cancer than in unselected PDAC cases (Table 2). The results of our study show that almost 2% of

Table 2 List of studies describing *PALB2* mutation analysis in probands with sporadic pancreatic adenocarcinoma (PDAC), familial PDAC, and breast cancer families with personal or familial pancreatic cancer history

Analyzed patients; (N)	*PALB2* mutation carriers; N (%)	Country	Study
Sporadic or unselected pancreatic cancer (7 *PALB2* mutation carriers out of 937 patients; 0.75%)			
201	1 (0.5)	Canada, USA	Zhen et al. 2014 (24)
180	0	Canada	Grant et al. 2014 (25)
152	3 (2.0)	Czech	Current study
114	0	Canada	Tischkowitz et al. 2009 (26)
100	3 (3.0)	Australia	Waddell et al. 2015 (27)
96	0	USA	Hu et al. 2015 (1)
94	0	USA	Norris et al. 2014 (28)
Familial pancreatic cancer (16 *PALB2* mutation carriers out of 1557 patients; 1.02%)			
638	5 (0.8)	North America	Roberts et al. 2016 (29)
515	3 (0.6)	Canada, USA	Zhen et al. 2014 (24)
101	1 (1.0)	Canada	Tischkowitz et al. 2009 (26)
96	3 (3.1)	USA	Jones et al. 2009 (23)
81 (*BRCA2* WT)	3 (3.7)	Europe	Slater et al. 2010 (30)
71	0	Canada	Grant et al. 2014 (25)
28 (*BRCA1/2* WT)	0	Dutch	Harinck et al. 2012 (31)
15	0	Italy	Ghiorzo et al. 2012 (32)
7	0	USA	Norris et al. 2014 (28)
5	1 (20.0)	Canada	Grant et al. 2013 (33)
Breast cancer families with personal or familial pancreatic cancer history (7 *PALB2* mutation carriers out of 545 patients; 1.28%)			
132 (*BRCA1/2* WT)	2 (1.5)	Spanish	Blanco et al. 2013 (34)
94 (*BRCA1/2* WT)	2 (2.1)	USA	Hofstatter et al. 2011 (35)
77 (*BRCA1/2* WT)	0	USA	Stadler et al. 2011 (36)
62 (*BRCA1/2* WT)	3 (4.8)	Italy	Peterlongo et al. 2011 (37)
45 (*BRCA1/2* WT)	0	Dutch	Adank et al. 2011 (16)
39	0	Canada	Grant et al. 2014 (25)
39	0	Canada	Tischkowitz et al. 2009 (26)
29	0	Italy	Ghiorzo et al. 2012 (38)
28 (*BRCA1/2* WT)	0	Dutch	Harinck et al. 2012 (31)

Abbreviation: BRCA1/2 WT, negatively tested for presence of hereditary pathogenic mutations in the *BRCA1* and *BRCA2* genes.

Czech unselected PDAC patients carried hereditary truncating mutations in *PALB2*. This represents low but clinically meaningful population of patients with increased risk of PDAC development.

The novel variant c.1838delA results in truncation of the entire conservative WD40 repeat of the PALB2 protein required for its interaction with BRCA2 (39). Two previously described truncating *PALB2* mutations (c.509_510delGA and c.697delG) were repeatedly described in various populations, as reviewed in (40). The variant c.509_510delGA belongs to the most frequent pathogenic *PALB2* mutations and c.697delG was identified in patients from South Africa and Italy (20,21).

None of the carriers identified in our study displayed an indicative history of familial PDAC; however, one patient had a mother with breast cancer diagnosed in 53 years of age which is in agreement with *PALB2* association with breast cancer development (41). Another patient had a father with gastric cancer in an older age. The association between hereditary *PALB2* mutations and increased risk of gastric cancer (including the hereditary diffuse gastric cancer syndrome) has been proposed recently (30,42). Unfortunately, we were not able to perform segregation analyses of *PALB2* mutations in these families as DNA sample were not available.

Interestingly, while median age at PDAC diagnosis in the US population was 72 years (2), our patients were younger and the carriers of *PALB2* mutations were significantly younger than non-carriers (50 years vs. 63 years; $P = 0.016$). The earlier age of PDAC onset was also shown in the study of Kim et al. analyzing US pancreatic cancer patients carrying hereditary mutations in other PDAC-susceptibility genes—*BRCA1* (mean age at diagnosis 63 years; $P = 0.0014$) and *BRCA2* (mean age at diagnosis 63 years; $P = 0.0011$) (43). The earlier onset of pancreatic cancer in US carriers of mutations in PDAC-susceptibility genes was also reported in the study of Salo-Mullen et al. showing that pathogenic hereditary mutations were found in 28.6% and 6.5% of patients diagnosed at the age of ≤50 years and ≥70 years, respectively (44).

Although the frequency of *PALB2* mutation carriers in our study is low, the lack of indicative family cancer history and younger age at diagnosis may indicate that the *PALB2* analysis should be offered not only to patients from pancreatic and breast cancer families but also to younger patients (at the age of diagnosis less than 60 years) irrespectively to their family cancer history. However, it is important to emphasize that the exact risk of PDAC associated with *PALB2* mutations has not been calculated yet due to the small number of identified carriers. Therefore, more studies analyzing *PALB2* mutations in PDAC patients in various populations and subsequent segregation analyses that will also show the association of *PALB2* mutations with other cancers are highly desirable to improve the PDAC risk estimates and clinical management in mutation carriers. Similar to *PALB2*, identification of germline mutations in other PDAC-susceptibility genes lack clear clinical guidelines that limits routine genetic management in PDAC patients nowadays. However, implementation of NGS technologies into clinical settings will help to identify larger population of carriers of mutations in PDAC susceptibility genes that occur with low population frequencies.

Unaffected relatives who carry the *PALB2* mutation may benefit from targeted reduction of preventable risk factors (e.g. smoking cessation, omitting of alcoholic beverages) and from extended screening for early cancer diagnosis which may improve a clinical management of PDAC. This screening should include endoscopic ultrasonography and/or MRI/magnetic resonance cholangiopancreatography (45). Besides the preventive screening for PDAC, the proper clinical management of increase breast cancer risk in female carriers should be considered.

Moreover, the results of genetic testing may also help to better select patients for available targeted therapies and to develop novel therapeutic approaches. While PDAC patients only poorly respond to a standard chemotherapy (such as gemcitabine), an improved survival has been reported for patients carrying germline *BRCA1* or *BRCA2* mutations who receive platinum-based therapy vs. those treated with non-platinum chemotherapies (overall survival 22 vs. 9 months; $P = 0.039$ for pancreatic cancer patients stage 3/4) (46). The personalized cancer treatment was shown in a patient with biallelic *PALB2* mutation identified by NGS in surgically-removed, advanced, gemcitabine-resistant PDAC tumor. The disease progression was successfully controlled by a consecutive treatment of DNA-damaging agents mitomycin C and cisplatin (active against xenografts generated from patient's surgically resected tumor) over the period of 3 years (47). Recently, the induced synthetic lethality by poly(ADP-ribose) polymerase inhibitors (PARPi) has been approved for the treatment of DNA repair-deficient high-grade ovarian tumors in *BRCA1* or *BRCA2* mutation carriers. The first promising example of a treatment response obtained with combination of gemcitabine and a PARPi iniparib in *BRCA2*-associated PDAC was shown by Fogelman et al. (48). Because PALB2 protein participates together with BRCA2 in the same DNA repair pathway, germline mutations in *PALB2* could therefore represent an attractive biomarker for the targeted therapy by PARPi (40).

Conclusion

Our study demonstrates that approximately 2% of unselected Czech PDAC patients carry a pathogenic mutation in the *PALB2* gene. The presence of mutation was significantly associated with earlier disease onset while positivity of family cancer history was seen only in one out of three mutation carriers. Further studies are needed for the clinical implementation of our results. The results indicate that the *PALB2* mutation analysis could be offered not only to pancreatic cancer patients from families with multiple pancreatic and/or breast cancer cases but also to younger patients without the indicative family cancer history.

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Conflict of interest

None.

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Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.cancergen.2016.03.003.

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Research paper

The c.657del5 variant in the *NBN* gene predisposes to pancreatic cancer

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is the sixth most frequent cancer type in the Czech Republic with a poor prognosis that could be improved by an early detection and subsequent surgical treatment combined with chemotherapy. Genetic factors play an important role in PDAC risk. We previously identified one PDAC patient harboring the Slavic founder deleterious mutation c.657del5 in the *NBN* gene, using a panel next-generation sequencing (NGS). A subsequent analysis of 241 unselected PDAC patients revealed other mutation carriers. The overall frequency of c.657del5 in unselected PDAC patients (5/241; 2.07%) significantly differed from that in non-cancer controls (2/915; 0.2%; P = 0.006). The result indicates that the *NBN* c.657del5 variant represents a novel PDAC-susceptibility allele increasing PDAC risk (OR = 9.7; 95% CI: 1.9 to 50.2). The increased risk of PDAC in follow-up recommendations for *NBN* mutation carriers should be considered if other studies also confirm an increased frequency of c.657del5 carriers in PDAC patients from other populations.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the sixth most frequent cancer type (with an incidence of 19.6/100,000 persons in 2013) and the fifth most frequent cause of cancer death in the Czech Republic (www.svod.cz). The prognosis of PDAC is poor with a 5-year survival of 7% and a median survival of 6 months (Siegel et al., 2015). Early detection and subsequent surgical treatment combined with chemotherapy can improve the 5-year survival up to 40% (Nakao et al., 2006). While population screening is not rational due to the low PDAC incidence, the identification of high-risk individuals, who may benefit from the available screening methods, is desirable.

A genetic predisposition is the major endogenous risk factor of PDAC development, together with chronic pancreatitis and diabetes mellitus (Becker et al., 2014). It has been estimated that 5–10% of PDAC patients have a positive family PDAC history. The genetic basis of most familial PDAC cases has not been explained yet; however, several PDAC-susceptibility genes have been identified, including genes (*BRCA1*, *BRCA2*, *PALB2*, *MLH1*, *MSH2*, *MSH6*, *PMS2*, *STK11*, *APC*, *CDKN2A*) associated with hereditary cancer syndromes (reviewed in (Becker et al., 2014)). The protein products of numerous PDAC-susceptibility genes are directly involved in DNA repair and the DNA damage response. The most prevalent mutations have been identified in *BRCA2* (up to 6% of patients and increasing PDAC risk 3.5-fold (Couch et al., 2007)) and *PALB2* (3% of patients (Jones et al., 2009)). Their protein products share a common functional role in the DNA double-strand break (DDSB) repair. The *NBN* gene encodes nibrin, a protein participating in the formation of the multiprotein MRN (*MRE11-RAD50-NBN*) complex, an inevitable sensor of DNA damage in the DDSB repair (Carney et al., 1998). Biallelic *NBN* mutations predispose to the autosomal recessive Nijmegen-breakage syndrome (NBS) characterized by chromosomal instability and an increased risk of lymphoid malignancies and other cancers (Varon et al., 1998). Heterozygous *NBN* mutations predispose to breast cancer (BC) (Gorski et al., 2003), non-Hodgkin lymphoma (Steffen et al., 2006), and prostate cancer (Cybulski et al., 2013); however, their role in PDAC predisposition has not been studied yet. The most frequent pathogenic mutation in NBS patients and *NBN*-associated cancers is the recurrent Slavic founder mutation c.657del5 (c.657_661delACAAA) (Varon et al., 2000).

The next-generation sequencing (NGS) technology introduced analyses of large gene collections into genetic analyses in patients

Abbreviation: PDAC, pancreatic ductal adenocarcinoma; BRCA1, breast cancer gene 1; BRCA2, breast cancer gene 2; PALB2, partner and localizer of BRCA2; MLH1, homolog of *Escherichia coli* MutL 1; MSH2, homolog of *E. coli* MutS 2; MSH6, homolog of *E. coli* MutS 6; PMS2, postmeiotic segregation increased; STK11, serine/threonine protein kinase 11; APC, adenomatous polyposis coli gene; CDKN2A, cyclin-dependent kinase inhibitor 2A; DDSB, DNA double-strand break; NBN, nibrin; MRN, MRE11/RAD50/NBN complex; NBS, Nijmegen breakage syndrome; NGS, next-generation sequencing; FFPE, formalin-fixed paraffin-embedded; HRM, high resolution melting; FET, Fischer's exact test; OR, odds ratio; CI, confidence interval; BC, breast cancer; CHEK2, checkpoint kinase 2; PARP, poly(ADP-ribose) polymerase.

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with cancer susceptibility. Among others, *NBN* is routinely analyzed in many cancer gene sequencing panels. Recently, we have performed a study of germline variants influencing the breast cancer susceptibility in high-risk breast cancer patients using the custom panel NGS (Lhota et al., 2016). We subsequently used the identical approach for the analysis of pancreatic cancer predisposition in a PDAC patient from multiple cancer family. We identified the c.657del5 germline mutation in the *NBN* gene in this patient. Therefore, we aimed to determine the frequency of c.657del5 in unselected Czech PDAC patients.

2. Materials and methods

2.1. Panel NGS analysis in a patient with pancreatic ductal adenocarcinoma

In order to identify possible germline pathogenic variant in PDAC-susceptibility genes, we performed custom panel NGS targeting 581 genes in a PDAC patient from multiple cancer family (Fig. 1). The NGS and bioinformatics analysis was performed as described previously (Lhota et al., 2016) and revealed germline c.657del5 *NBN* variant. The mutation was confirmed by Sanger sequencing from independent PCR amplified blood DNA sample. The presence of the c.657del5 *NBN* variant in deceased proband's sister with gastric cancer (Fig. 1) was analyzed in DNA isolated from FFPE tumor tissue using the Cobas DNA Sample Preparation Kit (Roche).

2.2. Patients with pancreatic ductal adenocarcinoma

We genotyped c.657del5 *NBN* variant in blood-isolated DNA samples from 241 unselected, histopathologically-verified PDAC patients, which included 152 samples from the National Institute of Public Health [median age at diagnosis: 63 years (ranged 40–82); 59 females] and 89 samples from the Department of Oncology, General University Hospital in Prague [median age at diagnosis: 64 years (ranged 38–84); 49 females]. Information about family history of cancer in c.657del5 carriers was gathered from medical records when available.

The control group included 915 non-cancer individuals and it had been described and genotyped previously. All patients and controls were of Slavic descent and of Czech origin. The study was approved by the local Ethical Committees and a written informed consent was obtained from all participants.

2.3. The NBN c.657del5 genotyping

The exon 6 of the *NBN* gene was analyzed by a high resolution melting (HRM; LightCycler 480; Roche) using HOT FirePol EvaGreen HRM Mix (Solis BioDyne). The primer sequences had been described previously (Mateju et al., 2012). The presence of c.657del5 was confirmed by sequencing.

2.4. Statistical analysis

The difference between groups was calculated using the Fisher exact test (FET).

3. Results

We analyzed a PDAC patient (diagnosed at 64 years) from multiple-cancer family and identified the c.657del5 *NBN* mutation using the panel NGS (Fig. 1). Except to this germline mutation, we found no other truncating variants in other known PDAC-susceptibility genes (*BRCA1*, *BRCA2*, *PALB2*, *MLH1*, *MSH2*, *MSH6*, *PMS2*, *STK11*, *APC*, *CDKN2A*). The presence of c.657del5 mutation was confirmed also in the proband's sister deceased from gastric cancer (Fig. 1).

In the subsequent analysis, we genotyped c.657del5 in other 241 unselected PDAC patients and found five mutation carriers among them (2.07%). Thus, the frequency of c.657del5 among PDAC patients was significantly higher than that in previously analyzed controls (2/915), suggesting that the carriers of c.657del5 have an increased risk of PDAC development (OR = 9.7; 95%CI: 1.9–50.2; $P_{FET} = 0.006$). A PDAC family history was documented in none of the five c.657del5 carriers from 241 unselected PDAC patients; however, one patient had family cancer history (a sister with gastric cancer), and another female patient suffered from a duplicity of BC (at 46 years) and PDAC

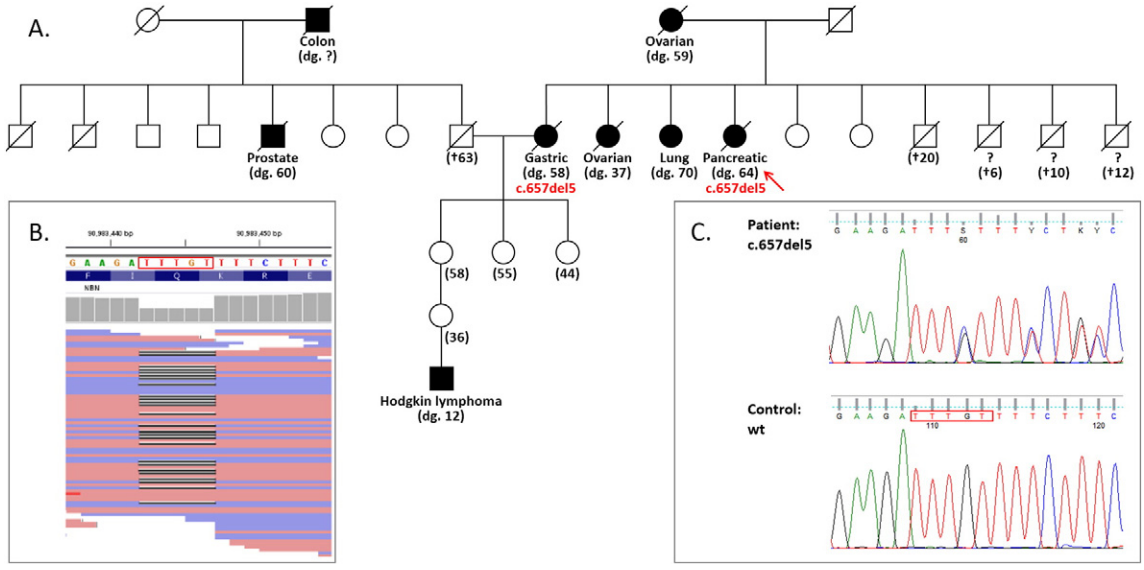

Fig. 1. Pedigree (A) of the multiple cancer family showing the proband with PDAC (indicated by an arrow) and her sister, both carrying c.657del5. DNA samples from other relatives were not available for genotyping. The ages of cancer diagnoses (dg.) or cessation (†) are indicated in the pedigree. The deletion of five nucleotides (TTTGT from reverse strand) is highlighted by a red frame in NGS analysis (B), confirmed by Sanger sequencing (C).

(at 64 years). The mean age at diagnosis for the c.657del5 carriers was 65.8 years (range 59–73).

4. Discussion

The highest frequency of *NBN* mutation carriers (up to 3.7% of patients) was found in BC patients from Central and Eastern Europe (Gorski et al., 2003). Recent meta-analysis indicated that c.657del5 is a moderate BC (OR = 2.51; 95%CI: 1.68–3.73) and lymphoma (OR = 2.93; 95%CI: 1.62–5.29) susceptibility allele, and that it also strongly increases the risk of prostate cancer (OR = 5.87; 95%CI: 2.51–13.75) (Gao et al., 2013). The association of the hereditary *NBN* mutations with BC susceptibility led to the inclusion of *NBN* into multigene cancer panel NGS analyses in high-risk individuals (Couch et al., 2015). Two studies have reported the results of hereditary mutation analysis performed by multi-gene panel testing in PDAC patients. While no truncating *NBN* mutation was identified in two previous studies of 290 and 638 patients, respectively (Grant et al., 2015; Roberts et al., 2016), Hu et al. found one c.657del5 carrier in 96 patients (also carrying the *CHEK2* mutation) (Hu et al., 2016). Recently, Lener et al. performed analysis of 10 prevalent founder mutations in *BRCA1*, *CHEK2*, *PALB2* and *NBN* (incl. c.657del5) in 383 pancreatic cancer patients and detected eight carriers of c.657del5 (2.09%), indicating the increased risk of pancreatic cancer in c.657del5 carriers (OR = 3.8; 95%CI: 1.68–8.60) in Poland (Lener et al., 2016).

The high frequencies of c.657del5 identified in PDAC patients in our and Lener et al. studies indicate that *NBN* is another DNA repair gene involved in PDAC-susceptibility. In comparison with our current study identifying 2.07% of c.657del5 carriers in unselected PDAC patients, earlier analyses found considerably lower frequencies of the mutation in Czech unselected BC (0.3%), colorectal cancer (0.3%), and lymphoma patients (0.8%) (Lhota et al., 2016; Pardini et al., 2009; Soucek et al., 2003). Our results and Lener et al. study (Lener et al., 2016) suggest that c.657del5 may be a novel PDAC-susceptibility allele significantly increasing the risk of PDAC development [combined OR calculated from this and Lener et al. studies comprising 624 pancreatic cancer patients (13 carriers of c.657del5) and 4915 controls (24 carriers) is 4.33; 95%CI 2.2–8.56; $p < 0.001$]. However, further studies in larger populations together with segregation analyses will be necessary to confirm our observation. They also may help specify the PDAC-associated risk more precisely, which is required for clinical management of the carriers and evaluation of c.657del5 as a putative predictive biomarker for therapy using DNA cross-linking agents or PARP inhibitors in carriers with PDAC (Schroder-Heurich et al., 2014).

Only the first patient identified in our preliminary NGS analysis had an indicative family cancer history (Fig. 1) and c.657del5 co-segregated with cancer diagnoses in the family. Its presence in the proband's sister with gastric cancer indicates that the carriers of c.657del5 may develop a broader spectrum of cancers. One in five carriers from the unselected PDAC group had a sister with gastric cancer (unfortunately, no DNA from this patient was available). The other mutation carriers displayed no family cancer history, just like the c.657del5 mutation carrier in the aforementioned report by Hu et al. (Hu et al., 2016). The similar mean age at PDAC diagnosis in carriers and non-carriers in our analysis (65.8 and 63.5 years, respectively) suggests that the c.657del5 mutation is not associated with an earlier disease onset.

In conclusion, our study suggests a novel role of the c.657del5 mutation in PDAC susceptibility. Future analyses of *NBN* in multi-gene cancer panels will help identify the hereditary pathogenic *NBN* mutations throughout the entire gene and enable a more accurate estimation of *NBN*-associated cancer risks.

Conflict of interest

None.

Acknowledgements

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ORIGINAL RESEARCH

Genetic analysis of subsequent second primary malignant neoplasms in long-term pancreatic cancer survivors suggests new potential hereditary genetic alterations

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Background: The principal aim of this report was to study second primary malignant neoplasms (SMNs) in long-term survivors of pancreatic ductal adenocarcinoma (PDAC) with regard to the germline genetic background.

Patients and methods: A total of 118 PDAC patients after a curative-intent surgery who were treated between 2006 and 2011 were analyzed. Of the 22 patients surviving for >5 years, six went on to develop SMNs. A genetic analysis of 219 hereditary cancer-predisposition and candidate genes was performed by targeted next-generation sequencing in germline DNA from 20 of these patients.

Results: Of all the radically resected PDAC patients, six patients went on to subsequently develop SMNs, which accounted for 27% of the long-term survivors. The median time to diagnosis of SMNs, which included two cases of rectal cancer, and one case each of prostate cancer, malignant melanoma, breast cancer, and urinary bladder cancer, was 52.5 months. At the time of analysis, none of these patients had died as a result of PDAC progression. We identified four carriers of germline pathogenic mutations in 20 analyzed long-term survivors. One carrier of the *CHEK2* mutation was found among four analyzed patients who developed SMNs. Of the remaining 16 long-term PDAC survivors, 3 patients (19%) carried germline mutation(s) in the *MLH1*+ *ATM*, *CHEK2*, and *RAD51D* gene, respectively.

Conclusion: This retrospective analysis indicates that SMNs in PDAC survivors are an important clinical problem and may be more common than has been acknowledged to be the case. In patients with good performance status, surgical therapy should be considered, as the SMNs often have a favorable prognosis.

Keywords: pancreatic ductal adenocarcinoma, second primary neoplasms, subsequent malignant neoplasm, hereditary cancer genes, long-term survivors, surgical treatment

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a malignant tumor with an extremely poor prognosis. Among radically operated patients in high-volume centers, five-year survival rates are as low as 4%–34%, with a median survival ranging between 17 and 27 months.[1]

Subsequent second primary malignant neoplasm (SMN) is a term used to describe a new primary cancer that occurs in a patient who has been diagnosed and treated for cancer in the past, months or years after the original primary cancer. SMNs are a major cause of mortality and serious morbidity among cancer survivors who have been

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successfully cured of their first cancer. Their etiologies are multiple and may relate to the role of primary cancer treatment (mainly radiotherapy and chemotherapy), unhealthy lifestyle behaviors, germline and somatic mutations, aging, and most likely a combination of any of these factors.[2,3] Because of the unfavorable prognosis, very few long-term PDAC survivors will develop SMN.[2,3] Consequently, there are very few reports about SMNs in PDAC survivors and their prognosis, and there is no information on the genetic background of these patients.[2–9]

The aim of the present study was to identify and describe SMNs in long-term PDAC survivors with regard to their potential genetic background. This is the first study describing the genetic background of long-term PDAC survivors with SMNs.

Patients and methods

Patients

This retrospective study involved 118 Caucasian patients with PDAC, who had undergone a curative-intent surgery between 2006 and 2011 at the University Hospital, Olomouc, Czech Republic.

The inclusion criteria for further SMN analysis included a curative-intent surgical treatment, histologic diagnosis of PDAC independently confirmed by two experienced pathologists, at least a five-year survival period after surgery, and postresection follow-up comprising biochemical tumor marker monitoring (CA 19-9, CEA, and CA 125) every 3 months and imaging (computed tomography [CT] or positron emission tomography [PET]/CT) scans performed every 6–12 months or in the case of CA 19-9 elevation.

The clinical data, including age, gender, date of diagnosis, pTNM stage,[10] the histologic type and grade of the tumor, lymphatic, vascular, and perineural invasion, the therapy administered and follow-up, were obtained from medical records. The main clinical characteristics of the whole group are summarized in Table 1. The retrospective study was approved by the Institutional Review Board of the University Hospital in Olomouc, and all living patients gave their informed written consent to participation in the study and the genetic analysis. The study was conducted in accordance with the Declaration of Helsinki.

The principal objective of this study was the identification of SMNs in this cohort of patients. The criteria used for the definition of SMN were derived from Waren and Gates, including a histologic confirmation of the second primary malignancy, anatomical separations of both tumors or recurrence exclusion, and a second tumor diagnosis >6 months after the diagnosis of the first tumor.[2] The SMNs in the studied cohort were diagnosed by physical examination, endoscopy, and/or diagnostic imaging (CT/PET-CT) and were histologically verified.

Table 1 Baseline patient characteristics (entire cohort)

Parameters	Number of patients*	%
Sex		
Male	75	64
Female	43	36
TNM stage		
I	20	17
IIA	34	29
IIB	54	46
III	2	2
IV	8	7
Histologic grade		
G1 + G2 (well to moderate)	62	52
G3 (poor)	51	44
Not available	5	4
Lymphovascular invasion		
pL0	74	63
pL1	38	32
Not available	6	5
Perineural invasion		
pP0	35	30
pP1	77	65
Not available	6	5
Angioinvasion		
pA0	91	77
pA1	21	18
Not assessed	6	5
Adjuvant therapy		
Yes	79	68
No	37	31
Unknown	2	2

Note: *118 patients in total.

Next-generation sequencing analysis

Blood was collected during diagnostic procedures using tubes with K_3EDTA anticoagulant, and DNA was isolated from lymphocytes using the phenol/chloroform extraction method described by Sugimura.[11]

A custom-designed CZECANCA panel (SeqCap EZ choice; Nimblegen/Roche) for the germline-targeted next-generation sequencing (NGS) analysis of cancer-predisposition and candidate genes was used as described previously.[12] In brief, the panel targets 219 selected genes with a known predisposition to hereditary cancer syndromes (including breast, ovarian, colorectal, pancreatic, gastric, endometrial, kidney, prostate, and skin cancers) and other genes that code for proteins involved in the DNA repair and/or DNA damage response with uncertain clinical relevance. A sequencing

library was prepared using the KAPA HTP Library Preparation kit according to the manufacturer's instructions (KAPA Biosystems, Roche) and sequenced on the MiSeq instrument with MiSeq reagent Kit v3 (Illumina).

Bioinformatics analysis

The NGS data were processed according to the in-house bioinformatics pipeline as described recently.[12] In brief, SAM files were generated from FASTQ files using Novoalign v2.08.03 and transformed into BAM files using Picard tools v1.129. The VCF files prepared by GATK were annotated by ANNOVAR.[13] Medium-size indel identification was based on the method of soft-clipped bases using Pindel software, and copy number variation (CNV) analysis was performed using CNV kit. During variant filtration, we excluded low-quality variants (sequence quality <30) and common variants with allelic frequencies >0.01 in ESP6500 and 1,000 genomes databases, respectively. We also excluded variants present >2× in a national database of genotypes that included 507 noncancer controls (data not shown). Nonsense, frameshift, and consensus dinucleotide splice site variants (±1/2) in known predisposition genes were classified as pathogenic or likely pathogenic. Missense variants, silent variants, in-frame indels, and other intronic variants were considered only when reaching a CADD score >2 and gerp >0 and classified according to the ClinVar and/or VarSome database. Prioritized variants were further analyzed by three prediction tools (SIFT, PolyPhen-2, and Mutation Analyzer). Variants predicted to be damaging by at least two programs were considered potentially deleterious.

Results

Patients and treatment

Twenty-two patients (19.1%) with histopathologically verified PDAC survived for >5 years since the primary PDAC diagnosis (long-term survivors) and matched the inclusion criteria for this retrospective study. The median follow-up was 6.2 years (range 5–11 years). Long-term PDAC survivors were further screened for the development of SMNs.

Overall, six patients (5.1% of all radically resected PDAC patients) developed SMNs. The SMN rate among long-term survivors was 27% (N=6/22). The mean age of the long-term PDAC survivors at the time of PDAC diagnosis was 61.7±7.8 years (range 44–75 years). The subgroup of patients with SMNs consisted of five males and only one female; the mean age was 66.7±7.4 years (range 51–75 years) at the time of PDAC diagnosis. None of these patients received neoadjuvant chemotherapy. One patient was treated with chemotherapy based on 5-fluorouracil (300 mg/m^2/day) concomitant to radiotherapy (50.4 Gy in 5.5 weeks) in the adjuvant setting, and the other five patients were treated with six 4-week cycles of gemcitabine (1000 mg/m^2 at days 1, 8, and 22). Overall, of the long-term PDAC survivors in the present cohort, around 40% of patients who received gemcitabine postoperatively developed subsequent malignant neoplasms. The clinical and pathologic data of the patients with SMN are summarized in Table 2.

Timing and patterns of subsequent secondary malignant neoplasms

The median time to SMN was 52.5 months (range 8.8–87.1 months; Table 2). The SMNs observed included two cases of rectal cancer, and one case each of prostate cancer, malignant melanoma, breast cancer, and urinary bladder cancer. Four of these patients underwent a curative surgery for the SMN. The patient with urinary bladder cancer underwent a radical cystectomy 63 months after PDAC resection. The patient with malignant melanoma underwent a radical excision 45.4 months after PDAC resection, and the patient with breast cancer underwent mastectomy 8.8 months after PDAC resection. All these patients are still alive with no recurrence of primary or secondary malignancy (6.3–8.9 years following the primary surgery of PDAC). One patient with rectal cancer died of postoperative complications from rectal surgery 64 months after the PDAC surgery. A second patient with rectal cancer died of cardiovascular comorbidities 62 months after the PDAC surgery without a specific therapy.

Prostate cancer with bone metastases was diagnosed in one patient 87.1 months after the primary PDAC resection and the patient was treated with hormonal therapy.

In summary, none of these patients died as a result of the PDAC.

Genetic analysis

A targeted NGS analysis covering 219 PDAC and other cancer susceptibility genes (Table 3) was performed in 20 patients both with and without SMNs (DNA samples from the two deceased patients with rectal cancer were not available).

Deleterious germline mutations were identified in 4 out of 20 NGS-analyzed long-term survivors (20%; Table 4). One patient harbored two deleterious mutations (in *MLH1* and *ATM*). Of the four sequenced long-term survivors who developed SMN, one female patient who developed breast cancer 1 year after primary PDAC diagnosis with no family cancer history carried a deleterious missense mutation in *CHEK2* (c.349A>G, p.Arg117Gly). Two out of 3 carriers of a

Table 2 Clinical data of patients with SMN

Sex	Age	pT	pN	Grade	Perineural invasion	Angioinvasion	Lymphovascular invasion	Adjuvant treatment	Family history of PDAC	Family history of other cancers	DFS	SMN	TTS	Treatment of SMN	TTT	OS	Status
Male	68	3	0	3	Yes	No	No	GEM	No	No	64	Rectal cancer	60	Surgery	60	64	Died
Male	69	2	I	3	No	No	No	GEM	No	No	105	Urinary bladder cancer	17	Surgery	63	105	Alive
Male	67	3	I	3	No	No	No	GEM	Yes	No	14	Malignant melanoma	45	Surgery	45	104	Alive
Male	51	3	0	2	Yes	No	Yes	GEM	No	No	92	Prostate cancer	87	Hormonal therapy	87	92	Alive
Male	75	2	0	I	No	No	No	R/5FU	No	No	62	Rectal cancer	61	None	NA	62	Died
Female	70	3	0	2	No	No	Yes	GEM	No	No	73	Breast cancer	9	Surgery	9	73	Alive

Abbreviations: pT, pathologic tumor size; pN, pathologic lymph node metastasis; DFS, disease-free survival (months); NA, not applicable; SMN, subsequent secondary malignant neoplasm; TTS, time to diagnosis of SMN (months); TTT, time to therapy of SMN (months); OS, overall survival (months); GEM, gemcitabine (six cycles); R/5 FU, concomitant chemoradiotherapy with 5-fluorouracil; PDAC, pancreatic ductal adenocarcinoma.

pathogenic mutation in 16 long-term PDAC survivors without SMN had a positive family cancer history. A patient with *RAD51D* splice-site mutation c.345+2T> G had a mother with gastric cancer and a patient with two mutations (nonsense variant in *MLH1*: c.390C>G and frame-shift variant in *ATM*: c.3849delA) had a father with a colorectal cancer and a father's mother with brain tumor. The remaining patient with the *CHEK2* c.1100delC mutation had no personal or family cancer history.

Subsequently, we identified several alterations with unknown impact on protein function. Fourteen variants in ten patients were predicted to be damaging by at least three prediction programs (Table 5).

Discussion

This report demonstrates a relatively high incidence of SMNs in five-year survivors of PDAC. The incidence of SMNs is generally 2%–10% and the prevalence is 6.6%–9%, accounting for about 16% of overall cancer incidence.[2,3,5] So far, very few publications have reported an analysis of second primary extrapancreatic malignancies following PDAC, probably because of the poor prognosis of these patients.[2,6–9] A large population-based study calculated the incidence of SMNs diagnosed after the diagnosis of PDAC to be lower when compared to other cancers (around 1.3%).[8,14] The latest report of the Czech National Cancer Registry shows a primary PDAC incidence of about 84% and a second primary PDAC (PDAC as the second primary tumor) incidence of about 16%. The incidence of synchronous PDAC and other malignancies is 5% of total PDAC patient incidence and the incidence of SMNs following PDAC is <1% of the total.[15] These rates were confirmed by the study reported by Hackert et al.[16]

The unexpectedly high number of SMNs (5%) in the present cohort of resected PDAC patients may be primarily explained by the comprehensive follow-up focusing not only on PDAC recurrence, but also on SMNs. Moreover, among long-term PDAC survivors, we identified SMNs in 27% of patients, indicating that the apparently limited number of SMNs in PDAC reported so far may be largely due to the poor prognosis. Previously published reports on long-term PDAC survivors show prevalences of SMNs ranging between 0% and 20%.[6,7] Nevertheless, this retrospective analysis may indicate that the development of SMNs in PDAC survivors may be more frequent than has been acknowledged in previous reports.

Improved medical options including anticancer therapy and treatment individualization lead to the prolongation of survival. This is evident in survivors of various primary

Table 3 List of genes analyzed by targeted next-generation sequencing

Abbreviation	Gene name (alternative denominations)
AIP	Aryl hydrocarbon receptor interacting protein
ALK	Anaplastic lymphoma kinase
APC	Adenomatous polyposis coli
APEX1	APEX nuclease (multifunctional DNA repair enzyme) 1
ATM	Ataxia telangiectasia mutated
ATMIN	ATM interactor
ATR	Ataxia telangiectasia and Rad3 related
ATRIP	ATR interacting protein
AURKA	Aurora kinase A
AXIN1	Axin 1
BABAM1	BRISC and BRCA1 A complex member 1
BAP1	BRCA1-associated protein-1 (ubiquitin carboxy-terminal hydrolase)
BARD1	BRCA1-associated RING domain 1
BLM	Bloom syndrome, RecQ helicase-like
BMPR1A	Bone morphogenetic protein receptor, type IA
BRAP	BRCA1-associated protein
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
BRCC3	BRCA1/BRCA2-containing complex, subunit 3
BRE	Brain and reproductive organ-expressed (TNFRSF1A modulator)
BRIP1	BRCA1 interacting protein C-terminal helicase 1
BUB1B	Budding uninhibited by benzimidazoles 1 homolog beta (yeast)
C11orf30	Chromosome 11 open reading frame 30 (EMSY)
C19orf40	Chromosome 19 open reading frame 40 (FAAP24)
CASP8	Caspase 8, apoptosis-related cysteine peptidase
CCND1	Cyclin D1
CDC73	Cell division cycle 73, Paf1/RNA polymerase II complex component, homolog (*Saccharomyces cerevisiae*)
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)
CDK4	Cyclin-dependent kinase 4
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)
CDKN1C	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha
CEP57	Centrosomal protein 57 kDa
CLSPN	Claspin
CSNK1D	Casein kinase 1, delta
CSNK1E	Casein kinase 1, epsilon
CWF19L2	CWF19-like 2, cell cycle control (*Schizosaccharomyces pombe*)
CYLD	Cylindromatosis (turban tumor syndrome)
DCLRE1C	DNA cross-link repair 1C
DDB2	Damage-specific DNA binding protein 2, 48 kDa
DHFR	Dihydrofolate reductase
DICER1	Dicer 1, ribonuclease type III
DMC1	DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (yeast)
DNAJC21	DnaJ (Hsp40) homolog, subfamily C, member 21
DPYD	Dihydropyrimidine dehydrogenase
EGFR	Epidermal growth factor receptor
EPCAM	Epithelial cell adhesion molecule
EPHX1	Epoxide hydrolase 1, microsomal (xenobiotic)
ERCC1	Excision repair cross-complementing rodent repair deficiency, complementation group 1
ERCC2	Excision repair cross-complementing rodent repair deficiency, complementation group 2
ERCC3	Excision repair cross-complementing rodent repair deficiency, complementation group 3
ERCC4	Excision repair cross-complementing rodent repair deficiency, complementation group 4
ERCC5	Excision repair cross-complementing rodent repair deficiency, complementation group 5
ERCC6	Excision repair cross-complementing rodent repair deficiency, complementation group 6
ESR1	Estrogen receptor 1

(*Continued*)

Table 3 (*Continued*)

Abbreviation	Gene name (alternative denominations)
ESR2	Estrogen receptor 2 (ER beta)
EXO1	Exonuclease 1
EXT1	Exostosin 1
EXT2	Exostosin 2
EYA2	Eyes absent homolog 2 (Drosophila)
EZH2	Enhancer of zeste homolog 2 (Drosophila)
FAM175A	Family with sequence similarity 175, member A
FAM175B	Family with sequence similarity 175, member B
FAN1	FANCD2/FANCI-associated nuclease 1
FANCA	Fanconi anemia, complementation group A
FANCB	Fanconi anemia, complementation group B
FANCC	Fanconi anemia, complementation group C
FANCD2	Fanconi anemia, complementation group D2
FANCE	Fanconi anemia, complementation group E
FANCF	Fanconi anemia, complementation group F
FANCG	Fanconi anemia, complementation group G
FANCI	Fanconi anemia, complementation group I
FANCL	Fanconi anemia, complementation group L
FANCM	Fanconi anemia, complementation group M
FBXW7	F-box and WD repeat domain containing 7, E3 ubiquitin protein ligase
FH	Fumarate hydratase
FLCN	Folliculin
GADD45A	Growth arrest and DNA-damage-inducible, alpha
GATA2	GATA binding protein 2
GPC3	Glypican 3
GRB7	Growth factor receptor-bound protein 7
HELQ	Helicase, POLQ-like
HNF1A	HNF1 homeobox A
HOXB13	Homeobox B13
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
HUS1	HUS1 checkpoint homolog (*S. pombe*)
CHEK1	Checkpoint kinase 1
CHEK2	Checkpoint kinase 2
KAT5	K(lysine) acetyltransferase 5
KCNJ5	Potassium inwardly rectifying channel, subfamily J, member 5
KIT	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
LIG1	Ligase I, DNA, ATP-dependent
LIG3	Ligase III, DNA, ATP-dependent
LIG4	Ligase IV, DNA, ATP-dependent
LMO1	LIM domain only 1 (rhombotin 1)
LRIG1	Leucine-rich repeats and immunoglobulin-like domains 1
MAX	MYC-associated factor X
MCPH1	Microcephalin 1
MDC1	Mediator of DNA-damage checkpoint 1
MDM2	Mdm2, p53 E3 ubiquitin protein ligase homolog (mouse)
MDM4	Mdm4 p53 binding protein homolog (mouse)
MEN1	Multiple endocrine neoplasia 1
MET	Met proto-oncogene (hepatocyte growth factor receptor)
MGMT	O-6-methylguanine-DNA methyltransferase
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (*Escherichia coli*)
MLH3	mutL homolog 3 (*E. coli*)
MMP8	Matrix metallopeptidase 8 (neutrophil collagenase)
MPL	Myeloproliferative leukemia virus oncogene
MRE11A	MRE11 meiotic recombination 11 homolog A (*S. cerevisiae*)
MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1 (*E. coli*)
MSH3	mutS homolog 3 (*E. coli*)

(*Continued*)

Table 3 (*Continued*)

Abbreviation	Gene name (alternative denominations)
MSH5	mutS homolog 5 (*E. coli*)
MSH6	mutS homolog 6 (*E. coli*)
MSR1	Macrophage scavenger receptor 1
MUS81	MUS81 endonuclease homolog (*S. cerevisiae*)
MUTYH	mutY homolog (*E. coli*)
NAT1	N-acetyltransferase 1 (arylamine N-acetyltransferase)
NBN	Nibrin
NCAM1	Neural cell adhesion molecule 1
NELFB	Cofactor of BRCA1
NF1	Neurofibromin 1
NF2	Neurofibromin 2 (merlin)
NFKBIZ	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta
NHEJ1	Nonhomologous end-joining factor 1
NSD1	Nuclear receptor binding SET domain protein 1
OGG1	8-oxoguanine DNA glycosylase
PALB2	Partner and localizer of BRCA2
PARP1	Poly (ADP-ribose) polymerase 1
PCNA	Proliferating cell nuclear antigen
PHB	Prohibitin
PHOX2B	Paired-like homeobox 2b
PIK3CG	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit gamma
PLA2G2A	Phospholipase A2, group IIA (platelets, synovial fluid)
PMS1	PMS1 postmeiotic segregation increased 1 (*S. cerevisiae*)
POLB	Polymerase (DNA directed), beta
POLD1	Polymerase (DNA directed), delta 1, catalytic subunit
POLE	Polymerase (DNA directed), epsilon, catalytic subunit
PPM1D	Protein phosphatase, Mg2+/Mn2+ dependent, 1D
PREX2	Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2
PRF1	Perforin 1 (pore forming protein)
PRKAR1A	Protein kinase, cAMP-dependent, regulatory, type I, alpha
PRKDC	Protein kinase, DNA-activated, catalytic polypeptide
PTEN	Phosphatase and tensin homolog
PTCH1	Patched 1
PTTG2	Pituitary tumor-transforming 2
RAD1	RAD1 homolog (*S. pombe*)
RAD17	RAD17 homolog (*S. pombe*)
RAD18	RAD18 homolog (*S. cerevisiae*)
RAD23B	RAD23 homolog B (*S. cerevisiae*)
RAD50	RAD50 homolog (*S. cerevisiae*)
RAD51	RAD51 homolog (*S. cerevisiae*)
RAD51AP1	RAD51 associated protein 1
RAD51B	RAD51 homolog B (*S. cerevisiae*)
RAD51C	RAD51 homolog C (*S. cerevisiae*)
RAD51D	RAD51 homolog D (*S. cerevisiae*)
RAD52	RAD52 homolog (*S. cerevisiae*)
RAD54B	RAD54 homolog B (*S. cerevisiae*)
RAD54L	RAD54-like (*S. cerevisiae*)
RAD9A	RAD9 homolog A (*S. pombe*)
RB1	Retinoblastoma 1
RBBP8	Retinoblastoma binding protein 8
RECQL	RecQ protein-like (DNA helicase Q1-like)
RECQL4	RecQ protein-like 4
RECQL5	RecQ protein-like 5
RET	Ret proto-oncogene
RFC1	Replication factor C (activator 1) 1, 145 kDa
RFC2	Replication factor C (activator 1) 2, 40 kDa

(*Continued*)

Table 3 (*Continued*)

Abbreviation	Gene name (alternative denominations)
RFC4	Replication factor C (activator 1) 4, 37 kDa
RHBDF2	Rhomboid 5 homolog 2 (Drosophila)
RNF146	Ring finger protein 146
RNF168	Ring finger protein 168, E3 ubiquitin protein ligase
RNF8	Ring finger protein 8, E3 ubiquitin protein ligase
RPA1	Replication protein A1, 70 kDa
RUNX1	Runt-related transcription factor 1
SDHAF2	Succinate dehydrogenase complex assembly factor 2
SDHB	Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)
SETBP1	SET binding protein 1
SETX	Senataxin
SHPRH	SNF2 histone linker PHD RING helicase, E3 ubiquitin protein ligase
SLX4	SLX4 structure-specific endonuclease subunit homolog (*S. cerevisiae*)
SMAD4	SMAD family member 4
SMARCA4	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 4
SMARCB1	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily b, member 1
SMARCE1	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily e, member 1
STK11	Serine/threonine kinase 11
SUFU	Suppressor of fused homolog (Drosophila)
TCL1A	T-cell leukemia/lymphoma 1A
TELO2	TEL2, telomere maintenance 2, homolog (*S. cerevisiae*)
TERF2	Telomeric repeat binding factor 2
TERT	Telomerase reverse transcriptase
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TMEM127	Transmembrane protein 127
TOPBP1	Topoisomerase (DNA) II binding protein 1
TP53	Tumor protein p53
TP53BP1	Tumor protein p53 binding protein 1
TSC1	Tuberous sclerosis 1
TSC2	Tuberous sclerosis 2
TSHR	Thyroid stimulating hormone receptor
UBE2A	Ubiquitin-conjugating enzyme E2A
UBE2B	Ubiquitin-conjugating enzyme E2B
UBE2I	Ubiquitin-conjugating enzyme E2I
UBE2V2	Ubiquitin-conjugating enzyme E2 variant 2
UBE4B	Ubiquitination factor E4B
UIMC1	Ubiquitin interaction motif containing 1
VHL	Von Hippel–Lindau tumor suppressor, E3 ubiquitin protein ligase
WRN	Werner syndrome, RecQ helicase-like
WT1	Wilms tumor 1
XPA	Xeroderma pigmentosum, complementation group A
XPC	Xeroderma pigmentosum, complementation group C
XRCC1	X-ray repair complementing defective repair in Chinese hamster cells 1
XRCC2	X-ray repair complementing defective repair in Chinese hamster cells 2
XRCC3	X-ray repair complementing defective repair in Chinese hamster cells 3
XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4
XRCC5	X-ray repair complementing defective repair in Chinese hamster cells 5
XRCC6	X-ray repair complementing defective repair in Chinese hamster cells 6
ZNF350	Zinc finger protein 350
ZNF365	Zinc finger protein 365

Table 4 Table of identified variants classified as likely pathogenic/pathogenic according to the ClinVar database

Patient	Gene	Nucleotide	Protein	ClinVar classification	Sex/age primary	Personal history (age at diagnosis)	Family history
With SMN							
OL0138	*CHEK2*	c.349A>G	p.Arg117Gly	Class 4–5	Female/70	Breast (71)	0
Without SMN							
OL0130	*RAD51D*	c.345+2T>G	–	Class 4	Male/62	0	Mother – gastric
OL0132	*MLH1*	c.390C>G	p.Tyr130Ter	Class 5	Female/52	0	Father – colon, father's mother – brain
	ATM	c.3849delA	p.Leu1283fs	Class 5			
PCI77	*CHEK2*	c.1100delC	p.Thr367fs	Class 5	Male/55	0	0

Note: All variants are heterozygous.
Abbreviation: SMN, subsequent malignant neoplasm after pancreatic ductal adenocarcinoma (PDAC).

Table 5 List of identified variants of unknown significance

Patient	Gene	Nucleotide	Protein	rs number	EXaC MAF	ClinVar/ VarSome classification	SIFT	PP2	MA	Damag. acc. to ≥2 software
With SMN										
OL0134	***BLM***	**c.11**T>C	**p.Val4Ala**	rs144706057	0.0017	1–3/3	**0**	0.132	**2.14**	Y
OL0135	***PTCH1***	**c.2597**G>A	**p.Gly866Glu**	NA	NA	3/3	0.08	**0.999**	**2.31**	Y
	ATM	c.3208G>A	p.Val1070Ile	NA	NA	3/3	0.35	0.026	**2.135**	N
OL0136	***PLA2G2A***	**c.185**G>A	**p.Arg62His**	NA	8.34E-05	NA/3	**0.02**	0.888	**3.005**	Y
	LRIG1	**c.2195**C>T	**p.Pro732Leu**	rs61746346	0.0022	NA/3	**0**	**0.991**	**1.975**	Y
	RECQL5	c.1801G>A	p.Val601Met	NA	NA	NA/3	0.3	0.04	**1.905**	N
OL0138	*PREX2*	c.C1672G	p.Pro558Ala	rs199541834	0.0001	NA/3	0.15	0.145	0.46	N
	PARP1	c.C659T	p.Ala220Val	rs139232092	0.0006	NA/3	0.15	0.003	1.155	N
Without SMN										
OL0041	*BUB1B*	c.1042G>A	p.Ala348Thr	NA	8.24E-06	NA/3	0.33	0.85	**2.175**	N
	MRE11A	c.C1475A	p.Ala492Asp	rs61749249	0.0034	1–3/3	0.43	0.754	1.735	N
OL0130	***XRCC1***	**c.632**A>G	**p.Tyr211Cys**	NA	1.74E-05	NA/3	0.15	**0.998**	**2.175**	Y
OL0131	*0*									
OL0132	***GRB7***	**c.1439**T>C	**p.Val480Ala**	rs143372931	0.0004	NA/3	**0**	0.848	**3.07**	Y
	RAD9A	c.215G>A	p.Arg72His	rs377299831	1.65E-05	NA/3	0.58	0.019	1.2	N
OL0133	***EXT2***	**c.1859**C>T	**p.Thr620Met**	rs138495222	0.0006	2–3/3	**0.02**	**0.999**	**2.24**	Y
	MLH3[a]	**c.**3281-1G>C	–	NA	NA	NA/3	–	–	–	–
OL0137	*PREX2*	c.2167A>G	p.Asn723Asp	NA	1.65E-05	NA/3	**0.03**	0.614	1.63	N
	HELQ	**c.1418**G>A	**p.Arg473His**	NA	2.48E-05	NA/3	**0**	**1**	**4.545**	Y
	RFC4	c.908C>T	p.Ala303Val	rs144238574	9.07E-05	NA/3	0.44	0.027	1.235	N
OL0139	*RHBDF2*	c.940G>A	p.Ala314Thr	rs140433374	0.0008	NA/3	0.33	**0.952**	1.78	N
	MDM4	c.1162C>G	p.Pro388Ala	rs61754765	0.0006	NA/3	0.92	**0.997**	1.1	N
OL0140	***FANCM***	**c.3407**T>C	**p.Leu1136Ser**	NA	1.65E-05	NA/3	**0.01**	**0.963**	**1.905**	Y
	POLE	**c.1601**T>C	**p.Leu534Pro**	NA	NA	NA/3	**0**	**0.991**	**3.565**	Y
OL0141	*0*									
OL0142	***RAD54L***	**c.1817**G>A	**p.Arg606Gln**	rs374574941	2.47E-05	NA/3	**0**	**1**	**4.735**	Y
	POLD1	**c.2116**C>G	**p.Pro706Ala**	NA	NA	3/3	**0.01**	0.733	**2.41**	Y
OL0144	*CWF19L2*	c.2240A>C	p.Lys747Ther	NA	NA	NA/3	0.08	0.697	**1.915**	N
	SETX	**c.967**A>G	**p.Ser323Gly**	NA	1.65E-05	NA/3	**0**	**0.994**	0.975	Y
OL0157	*TP53BP1*	c.2226A>T	p.Glu742Asp	rs150423877	0.0004	NA/3	0.48	**0.987**	0.46	N
PCI77	*0*									
PCI15	*PTCH1*	c.3376G>A	p.Val1126Ile	rs147025073	0.0005	3/3	0.26	**0.927**	1.77	N
	NCAM1	c.1481C>A	p.Thr494Asn	NA	NA	NA/3	**0.01**	0.347	NA	N
PCI39	*0*									
PCO11	*BRCA1*	c.3929C>A	p.Thr1310Lys	rs80357257	8.24E-06	1–3/3	**0.01**	0.787	1.895	N
	AURKA	c.1028G>A	p.Arg343Gln	rs200181472	0.0002	NA/3	**0.04**	0.027	0.71	N
	EXO1	c.820G>A	p.Gly274Arg	rs149397534	0.0021	NA/3	0.16	**0.999**	1.295	N

Notes: The variants predicted to be damaging by at least two out of three prediction tools employed are represented in bold. [a]The splice-site variant was analyzed by splicing prediction software spidex with a score –25.6359, suggesting that it is the damaging variant.
Abbreviation: NA, not applicable.

cancers, including PDAC survivors.[17] The same trend has also been confirmed in the Czech population.[18] A higher age at the time of the primary PDAC diagnosis was the only remarkable difference between five-year survivors with SMNs and those without SMNs. The incidence of cancer increases with age, and, consequently, older survivors have a higher risk of SMNs than younger survivors. All patients with a manifestation of SMN received adjuvant chemotherapy consisting of antimetabolites gemcitabine or 5-fluorouracil. Although patients who undergo chemotherapy are generally considered to be at a higher risk of SMN, an increased risk of SMNs after the use of these antimetabolites has not been reported to date.

Therefore, it seems that a higher age at the time of the PDAC diagnosis and a long-term survival after a surgical and chemotherapy treatment may be regarded as risk factors for SMNs, and that such patients should be diagnostically followed.

The NGS analysis revealed five clearly pathogenic variants in four patients from the long-term PDAC survivors subgroup (25%). This frequency was higher than for the other group of 96 unselected PDAC patients,[19] which was 13.5% identified with a panel of 22 genes, but we are aware of the small number of patients analyzed in our study. A recent study by Yurgelun et al[20] identified 28 carriers of germline pathogenic or likely pathogenic mutations in double-strand DNA damage repair genes in 289 patients (9.7%) with resected PDAC. Interestingly, the authors demonstrated that the germline mutations carriers had superior overall survival (HR 0.54; $P = 0.05$). This indicates that mutations in cancer-predisposing genes increase the risk of prognostically beneficial PDAC; therefore, it might be expected that an increased proportion of mutation carriers should also be found among the long-term PDAC survivors. Unfortunately, the genetic aberrations discovered do not currently seem to be of any clinical relevance with regard to potential therapeutic options.

Considering the small number of long-term survivors, the frequency of pathogenic variants in the group of patients who developed SMNs (25%) and in the group who did not (19%) was comparable. These results suggest that SMN development may be due to a combined effect of variants with low penetrance or may be caused by a combination of genetic and/or nongenetic risk factors. On the other hand, the presence of germline mutations did not dramatically influence risk and prognosis of SMN.

The patient with PDAC at 70 years old and subsequent breast cancer at 71 was identified to harbor a pathogenic missense *CHEK2* variant (c.349A>G, p.Arg117Gly). Numerous studies and meta-analyses have shown that mutations in the *CHEK2* gene are clearly associated with increased breast cancer risk and also with the development of other solid or hematologic tumors.[21] We failed to find a significant association of *CHEK2* germline variants with unselected PDAC cases in our previous study; however, only selected portions of *CHEK2* coding sequence were analyzed.[22] Since then, germline *CHEK2* mutations have been identified in several studies in patients with PDAC;[19,20,23,24] however, a consensual evaluation of *CHEK2* germline variants in PDAC remains to be established.

In a subgroup of 16 long-term PDAC survivors without SMN development, we identified 2 PDAC patients with pathogenic variants in cancer predisposition genes and a positive family history. *MLH1* is a Lynch syndrome predisposition gene[25] and can explain the colorectal cancer in the patient's father. *RAD51D* is an ovarian cancer predisposition gene,[26] but was never associated with gastric cancer. These data indicate that germline mutations in cancer predisposition genes are associated with a wider range of phenotypes than previously suggested.

The evaluation of potentially pathogenic missense germline variants in candidate genes requires further analysis in larger groups of PDAC patients, as well as functional studies, because in silico predictions are suitable for variant prioritization for such analyses, but are not devoted to final variant classification.

The present study, therefore, poses new questions regarding the role of genetic alterations in the development of PDAC and subsequent SMNs in patients, and regarding the modification of the clinical course of the disease. The variants identified in the present study must be verified by further investigations, also in regard to the functional impact. However, this is the first study of genetic alterations in SMNs in PDAC patients and the largest epidemiologic retrospective analysis of SMNs after PDAC treatment in Central Europe.

Conclusion

In our cohort, 27% of five-year PDAC survivors went on to develop SMNs. An intensive follow-up can identify the second primary neoplasms early, at a curable stage. SMN risk factors include a longer survival and a higher age at the time of PDAC diagnosis. Genetic analysis has confirmed the role of pathogenic mutations in pancreatic and other cancers' predisposition genes in long-term surviving PDAC patients; nevertheless, the frequency did not differ in the subgroups with and without SMN development. If the performance status of these patients allows and a second primary tumor

has a favorable prognosis, subsequent surgery should be performed.

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