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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	1	3	No	No	No	GEM	No	No	105	Urinary bladder cancer	17	Surgery	63	105	Alive
Male	67	3	1	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	1	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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