Genetic evaluation of patients with multiple primary cancers

MARIA VALERIA FREIRE[1], ROMAIN THISSEN[1], MARIE MARTIN[2], CORINNE FASQUELLE[2], LAURA HELOU[1], KEITH DURKIN[1,2], MARIA ARTESI[1,2], AIMÉ LUMAKA[1,2], NATACHA LEROI[2], KARIN SEGERS[2], MICHELLE DEBERG[2], JEAN-STÉPHANE GATOT[2], LIONEL HABRAN[3], LEONOR PALMEIRA[2], CLAIRE JOSSE[4] and VINCENT BOURS[1,2]

[1]Department of Human Genetics, GIGA Research Center-University of Liège and CHU Liège, 4000 Liège, Belgium; [2]Department of Human Genetics, CHU Liège, 4000 Liège, Belgium; [3]Department of Pathology, CHU Liège, 4000 Liège, Belgium; [4]Department of Medical Oncology, GIGA Research Center-University of Liège and CHU Liège, 4000 Liège, Belgium

Received April 10, 2024; Accepted August 22, 2024

DOI: 10.3892/ol.2024.14750

Abstract. Regarding inherited cancer predisposition, single gene carriers of pathogenic variants (PVs) have been extensively reported on in the literature, whereas the oligogenic coinheritance of heterozygous PVs in cancer-related genes is a poorly studied event. Currently, due to the increased number of cancer survivors, the probability of patients presenting with multiple primary cancers (MPCs) is higher. The present study included patients with MPCs aged ≤45 years without known PVs in common cancer predisposition genes. This study used whole exome sequencing (WES) of germline and tumoral DNA, chromosomal microarray analysis (CMA) of germline DNA (patients 1-7, 9 and 10), and a karyotype test of patient 8 to detect variants associated with the disease. The 10 patients included in the study presented a mean of 3 cancers per patient. CMA showed two microduplications and one microdeletion, while WES of the germline DNA identified 1-3 single nucleotide variants of potential interest to the disease in each patient and two additional copy number variants. Most of the identified variants were classified as variants of uncertain significance. The mapping of the germline variants into their pathways showed a possible additive effect of these as the cause of the cancer. A total of 12 somatic samples from 5 patients were available for sequencing. All of the germline variants were also present in the somatic samples, while no second hits were identified in the same genes. The sequencing of patients with early cancers, family history and multiple tumors is already a standard of care. However, growing evidence has suggested that the assessment of patients should not stop at the identification of one PV in a cancer predisposition gene.

Correspondence to: Professor Vincent Bours, Department of Human Genetics, CHU Liège, 1 Avenue de l'Hôpital, 4000 Liège, Belgium
E-mail: vbours@uliege.be

Key words: multiple primary cancers, early cancers, whole exome sequencing, cancer predisposition genes, single nucleotide variants

Introduction

Multiple primary cancers (MPCs) are defined as the occurrence of more than one synchronous or metachronous cancer in a patient. For reporting purposes, the identification of MPCs is variable across the regulatory agencies. The Surveillance, Epidemiology and End Results (SEER) program considers the histology, site, laterality, and time since initial diagnosis of the tumor, while for the International Agency for Research on Cancer (IARC) only one tumor is registered for an organ, irrespective of time. The definition of synchronous and metachronous tumors also varies, having a threshold of 2 months in SEER and 6 months in IARC (1,2).

The development of screening tests and new cancer therapies improved cancer patient survival. By 2005, almost 900,000 of the 11 million cancer survivors were diagnosed with more than one cancer. The risk of developing subsequent cancers varies with regards to the type of first primary site, age at diagnosis, environmental exposure and genetic factors (3).

The identification of genes associated with hereditary cancer risk started 25 years ago with the discovery of BRCA pathogenic variants in families with breast and ovarian cancers. Since then, more than 80 cancer predisposition syndromes and 100 cancer-related genes were identified (4). For each syndrome, the most commonly associated cancers are defined but the exact risk profile remains often unknown. One common characteristic of these conditions is the young age at cancer diagnosis in most patients, although a considerable heterogeneity is observed. Additionally, it is widely stated that patients with multiple primary cancers are more likely to carry germline pathogenic variants in cancer-related genes (5).

For patients with MPCs without a germline molecular genetic diagnosis after initial evaluation, single-nucleotide variants (SNVs), copy number variants (CNVs), or de novo chromosomal rearrangements could be the cause of the disease. Another possibility could be the additive effect of multiple genetic events influencing a single biochemical mechanism. The aim of this study was to identify novel genetic mechanisms associated with risk of tumor development in patients with MPCs.

Patients and methods

Patient selection. We selected patients with ≥2 tumors before 45 years old, who were evaluated by the genetic department of the CHU de Liège from March 2019 to December 2022. The routine genetic assessment and testing with targeted next-generation sequencing (NGS) failed to establish a germline molecular genetic diagnosis. Additionally, the patients lacked family history suggestive of a cancer predisposition syndrome. Tumors in the same tissue or organ were considered separate primary tumors if, in the case of paired organs, they presented bilaterally or if the clinical history clearly indicated that they were different. All participants gave informed consent to participate in the study.

Data collection. Patient sex, age, age at diagnosis of each of the tumors, personal and family history were extracted from the medical records. Data regarding cancer diagnosis and treatment was collected from the institutions database.

Germline analysis. Whole exome sequencing (WES, Novogene) and a high-resolution (180K and 60K) chromosomal microarray analysis reference (CMA, Agilent) were performed on the DNA extracted from the blood of the patients. The WES data was analyzed using our own Humanomics pipeline [as described in (6)] and two interpretation software (Illumina BaseSpace Variant Interpreter and Diploid Moon). The variants were filtered according to their quality (allele depth and genotype quality), population frequency, the effect on the gene and the classification of the variant in databases. Variant classification was performed according to ACMG Standards and Guidelines for the Interpretation of Sequence Variants (7). The variants identified in the WES analysis were confirmed by Sanger sequencing (Fig. S1).

CNV analysis was conducted on the BAM file (mapping with bwa *version v0.7.17* and cleaning with elprep *version v4.1.5*) arising from the identical sequencing batch using the Humanomics BED file, which enabled the detection of CNVs bigger than one whole exon. The depth of coverage was calculated specifically on exons within these files, and the normalization set comprised 400 patients (200 males and 200 females). The CNVs were detected by implementing of our in-house pipeline (StueckFinder v2.2) that integrates the well-established tool CANOES (8) and code modifications to enable CNV detection on the gonosomes. Variant classification was performed according to the ACMG/ClinGen technical standards for the interpretation and reporting of constitutional copy number variants (9). The detected CNVs were confirmed by nanopore sequencing.

Tumor analysis. WES of the tumoral DNA (using Mechanical Fragmentation and the Twist Universal Adapter System) was performed on the DNA extracted from the available Formalin-Fixed Paraffin-Embedded (FFPE) patient's samples. The data analysis was performed using QIAGEN Digital Insight Software Genomics Workbench 21 for data preparation, mapping, variant calling and annotation. The tumoral WES results were analyzed separately and as a tumor-normal pair. Variant classification was performed according to ACMG Standards and Guidelines for the Interpretation of Sequence Variants in Cancer (10) and the ClinGen specification when available. Mutational signatures in the samples were analyzed Using Mutational Patterns R package (11) and COSMIC v2 signatures (12), taking only the somatic variants into account.

Results

Patients' characteristics. Ten patients with multiple cancers were included in the study. The mean age was 40.7±5.4 years, 7 patients were female and 3 were male. None of the patients had family cancer history suggestive of a cancer predisposition syndrome. Before enrolment, by targeted NGS sequencing a *CHEK2* and *ATM* variant was identified in two patients, however they could not explain the full phenotype observed. The CMA results included two microduplications and one microdeletion (Table I).

We observed a mean of 3.2 tumors per patient. The tumors included melanomas, seminomas, thyroid cancer, gynecological tumors, and others (Table II).

Germline WES results. CNVs and missense, nonsense and splicing SNVs were identified in the patients included in the study (Table III). The variants were classified according to the ACMG criteria and ClinGen specifications whenever available. Most of the identified SNVs were variants of uncertain significance (VUS), and only two variants were classified as pathogenic (Table IV). One of the pathogenic variants, an *ATM* splicing variant (c.8988-1G>A) that affected a canonical splice site, was not present in GnomAD, was predicted as deleterious by SIFT, Mutation taster, Provean, and the splicing in silico analysis. The second pathogenic variant in the *MUTYH* gene was previously described in patients with *MUTYH*-Associated Polyposis, an autosomal recessive disease that predisposes to colorectal cancer (13). Furthermore, heterozygous deleterious *MUTYH* variants were described as drivers in various types of cancer (adrenocortical carcinoma, esophageal carcinoma, sarcoma, prostate adenocarcinoma and kidney renal clear cell carcinoma) (14).

Two CNVs were detected when evaluating WES data in the patients (Table III). One included a *MSR1* gene heterozygous deletion of exons 7-10. This CNV was classified as variant of unknow significance according to the ACMG/ClinGen criteria. The second was a heterozygous deletion of the whole *APOBEC3B* gene, which was classified as pathogenic as for a full gene deletion a pathogenic classification is warranted (15).

Tumor WES results. Twelve somatic samples were available for sequencing, a seminoma and thyroid cancer from patient 1, four melanomas from patient 3, an ovary cancer from patient 4, a thyroid cancer, dermatofibroma and dysplastic nevus from patient 6, and breast and thyroid cancer from patient 7. All the germline variants were also present in the somatic samples, no second hits were identified in the same genes. The mutational signature analysis aimed to identify common etiological factors for the development of the multiple cancers in the patients. The most frequently presented Single Base Substitution (SBS) signatures with a proposed etiology were the DNA mismatch repair alteration signature, and the signatures related to chemotherapy treatment (Table SI). The

Table I. Patient characteristics.

Patient	Sex	Age, years	Family cancer history and age at diagnosis, years	Cancer type and age at diagnosis, years	Initial genetic evaluation	CMA results
P1	M	44	Choroidal melanoma in father at 68	Melanoma at 18, left seminoma at 21, right seminoma at 37, thyroid cancer at 38	Kit BRCA HEREDITARY CANCER MASTR Plus: *BRCA1*, *BRCA2*, *PALB2*, *CHEK2*, *BARD1*, *BRIP1*, *RAD51C*, *RAD51D*, *TP53*, *MRE11A*, *RAD50*, *NBN*, *FAM175A*, *ATM*, *STK11*, *MEN1*, *PTEN*, *CDH1*, *MUTYH*, *BLM*, *XRCC2*, *MLH1*, *MSH6*, *PMS2* and *MSH2*, and the 3' UTR of *EPCAM*	arr[hg19] 2q13(111,408, 390-113,098, 686)x1
P2	F	43	Basal cell carcinoma at 37	Bilateral breast tumor at 23, melanoma at unknown age	Kit BRCA HEREDITARY CANCER MASTR Plus: *BRCA1*, *BRCA2*, *PALB2*, *CHEK2*, *BARD1*, *BRIP1*, *RAD51C*, *RAD51D*, *TP53*, *MRE11A*, *RAD50*, *NBN*, *FAM175A*, *ATM*, *STK11*, *MEN1*, *PTEN*, *CDH1*, *MUTYH*, *BLM*, *XRCC2*, *MLH1*, *MSH6*, *PMS2* and *MSH2*, and the 3' UTR of *EPCAM*	arr[hg19] 16p13.11(14910205-16525348)x3
P3	F	38	Generalized cancer in half-sister at 35	Melanoma at 23, 24, 30, 34 and 35	Sanger sequencing of *CDK4* and *BAP1*, direct sequencing of c.1100delC in gene *CHEK2*. High-throughput sequencing: BRCA MASTR Dx kit (*BRCA1*, *BRCA2*), *PALB2*, *TP53* (exons 2-11), *ATM*, *CDKN2A*	arr[hg19] (1-22, X)x2
P4	F	33	Pancreatic cancer in paternal grandmother at 64	Medulloblastoma at 10, ovary tumor at 26	Sanger sequencing of *CDK4* and *BAP1*, direct sequencing of c.1100delC in gene *CHEK2*. High-throughput sequencing: BRCA MASTR Dx kit (*BRCA1*, *BRCA2*), *PALB2*, *TP53* (exons 2-11), *ATM*, *CDKN2A*	arr[hg19] (1-22, X)x2
P5	M	42	No history	Colorectal cancer at 37, melanoma at unknown age	Kit HNPCC MASTR Plus: *MLH1*, *MSH2*, *MSH6*, *PMS2*, and 3' UTR of *EPCAM*. Sequencing of *APC*, *MTYH*, *NTHL1*, *TP53*, *BAP1*	arr[hg19] (1-22)x2, (XY)x1
P6	F	36	Thyroid cancer in sister at unknown age, breast cancer in mother after the age of 50 and maternal grandmother	Thyroid tumor at 26, melanoma at 28	Kit SUREMASTR HEREDITARY CANCER: *BRCA1*, *BRCA2*, *PALB2*, *TP53*, *CHEK2*, *MLH1*, *MSH2*, *MSH6*, *ATM*, *BRIP1*, *RAD51C*, *RAD51D*	arr[hg19] (1-22, X)x2
P7	F	44	Ovarian cancer in mother at 45, breast cancer in half-sister (same mother) at 37	Breast tumor at 35, thyroid tumor at 36	Kit BRCA HEREDITARY CANCER MASTR Plus: *BRCA1*, *BRCA2*, *PALB2*, *CHEK2*, *BARD1*, *BRIP1*, *RAD51C*, *RAD51D*, *TP53*, *MRE11A*, *RAD50*, *NBN*, *FAM175A*, *ATM*, *STK11*, *MEN1*, *PTEN*, *CDH1*, *MUTYH*, *BLM*, *XRCC2*, *MLH1*, *MSH6*, *PMS2* and *MSH2*, and the 3' UTR of *EPCAM*	arr[hg19] (1-22, X)x2
P8	F	52	Prostate cancer at 57, pheochromocytoma at 68 and paraganglioma at unknown age in father, breast cancer in mother at 49 and grandmother at 60	Breast tumor at 33, thyroid cancer at 38	High-throughput sequencing: Kit SUREMASTR HEREDITARY CANCER (*BRCA1*, *BRCA2*, *PALB2*, *TP53*, *CHEK2*, *MLH1*, *MSH2*, *MSH6*, *ATM*, *BRIP1*, *RAD51C*, *RAD51D*). *PTEN* gene sequencing and MLPA	arr[hg19] (1-22, X)x2

Table I. Continued.

Patient	Sex	Age, years	Family cancer history and age at diagnosis, years	Cancer type and age at diagnosis, years	Initial genetic evaluation	CMA results
P9	F	38	Synovial sarcoma in mother at 35	Melanoma at 31, thyroid cancer at 34	Arrhythmia/Primary Electrical disease panel: *ABCB4*, *ABCC9*, *ACTN2*, *AKAP9*, *ANK2*, *CACNA1C*, *CACNA2D1*, *CACNB2*, *CALM1*, *CALM2*, *CALM3*, *CASO2*, *CAY3*, *CTNNA3*, *DES*, *DPP6*, *DSC2*, *DSG2*, *DSP*, *GJA1 (CX43)*, *GJAS (CX40)*, *GNB5*, *GPD1L*, *HCN4*, *JUP*, *KCNAS*, *KCND3*, *KCNE1*, *KCNE2*, *KCNE3*, *KCNE5 (KCNE1L)*, *KCNH2*, *KCNJ2*, *KCNJ5 (GIRK4)*, *KCNJ8*, *KCNKI7*, *KCNQI*, *LMNA*, *NKX2-5 (NKX2E)*, *NOS1AP*, *NPPA*, *PKP2*, *PLN*, *PPA2*, *PRKAG2*, *RANGRF (MOG1)*, *RRAD*, *RYR2*, *SCN1B*, *SCN2B*, *SCN3B*, *SCN4B*, *SCN5A*, *SCN10A*, *SLMAP*, *SNTA1*, *TGFB3*, *TMEM43*, *TRDN*, *TRPM4*	arr[hg19] (1-22, X)x2
P10	M	37	No history	Seminoma at 24, bladder cancer at 33, kidney cancer at 34	High-throughput sequencing: Kit SUREMASTR HEREDITARY CANCER (*BRCA1*, *BRCA2*, *PALB2*, *TP53*, *CHEK2*, *MLH1*, *MSH2*, *MSH6*, *ATM*, *BRIP1*, *RAD51C*, *RAD51D*). *FH* gene sequencing. Hereditary pheochromocytoma and paraganglioma panel (*SDHA*, *SDHB*, *SDHC*, *SDHD*, *VHL*, *RET*). Hereditary non-polyposis colorectal cancer panel (*MLH1*, *MSH2*, *MSH6*). MLPA of *BRCA1*, *BRCA2*, *FH*, *SDHB*, *SDHC*, *SDHD*, *VHL*, *MLH1*, *MSH2*, *MSH6*	arr[hg19] 2q13(110862477-110964737)x4

CMA, chromosomal microarray analysis; F, female; M, male.

Table II. Tumor characteristics.

Characteristic	First tumor (n=10)	Second tumor (n=10)	Third tumor (n=6)	Fourth tumor (n=4)	Fifth tumor (n=2)	Total (n=32)
Mean ± SD age at presentation, years	26.9±8.5	30.8±6.0	33.8±4.0	35.3±1.5	35±1.4	
Tumor type, n (%)						
Melanoma	3 (30.0)	3 (30.0)	3 (50.0)	2 (50.0)	2 (100.0)	13 (40.6)
Seminoma	1 (10.0)	1 (10.0)	1 (16.7)	0 (0.0)	0 (0.0)	3 (9.4)
Thyroid cancer	1 (10.0)	3 (30.0)	0 (0.0)	1 (25.0)	0 (0.0)	5 (15.6)
Breast tumor	3 (30.0)	1 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (12.5)
Ovarian tumor	0 (0.0)	1 (10.0)	1 (16.7)	0 (0.0)	0 (0.0)	2 (6.3)
Neuronal tumors	1 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.1)
Muscle tumors	0 (0.0)	0 (0.0)	0 (0.0)	1 (25.0)	0 (0.0)	1 (3.1)
CRC	1 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.1)
Renal and urinary tract tumors	0 (0.0)	1 (10.0)	1 (16.6)	0 (0.0)	0 (0.0)	2 (6.3)

CRC, colorectal cancer; SD, standard deviation.

Table III. Genetic alterations identified in the patients.

Patient	Gene	Variant	Type
P1	*CHEK2*	c1100delC (p.Thr367Metfs*15)	Nonsense SNV
	EME2	c.964C>T (p.Gln322*)	Nonsense SNV
	ATRIP	c.1637T>G (p.Leu546Trp)	Missense SNV
	BUB1	arr[hg19] 2q13(111,408,390-113,098,686)x1	CNV
P2	*HNF1A*	c.92G>A (p.Gly31Asp)	Missense SNV
	TSC2	c.5383C>T (p.Arg1795Cys)	Missense SNV
	MSR1	c.(956+1_957-1)_ 3618del	CNV
P3	*ATM*	c.8988-1G>A	Splicing SNV
	APC	c.295C>T (p.Arg99Trp)	Missense SNV
P5	*ERCC2*	c.2260G>C (p.Glu754Gln)	Missense SNV
P6	*RASA2*	c.865T>C (p.Tyr289His)	Missense SNV
P7	*RIF1*	c.1475A>G (c.1475A>G)	Missense SNV
	APOBEC3B	g.(?_ 39378444)_(39388168_?)del	CNV
P8	*CHEK2*	c.434G>A (p.Arg145Gln)	Missense SNV
P9	*ATM*	c.2057T>A (p.Leu686His)	Missense SNV
P10	*MUTYH*	c.536A>G (p.Tyr179Cys)	Missense SNV

Reference transcripts: APC NM_000038.6, APOBEC3B NC_000081.7, ATM NM_000051.3, ATRIP NM_130384.2, BRCA1 NM_007294.3, CHEK2 NM_007194.4, EME2 NM_001257370.2, ERCC2 NM_000400.4, HNF1A NM_000545.5, MSR1 NM_138715.2, MUTYH NM_012222.2, RASA2 NM_006506.5, RIF1 NM_018151.4, TSC2 NM_000548.5.

only obvious correlation between the patient's signatures was observed between three of the four patient 3 melanomas.

Discussion

In this study only two out of 10 patients with MPCs presented clearly pathogenic SNVs. Previous reports showed similar results. When performing a whole-genome sequencing (WGS) of a cohort of patients with MPCs who had undergone genetic assessment, undetected germline pathogenic variants were identified in 15.2% cases (16). In another study, 21% of patients with MPCs had at least one PV identified (17). The rest of our patients had one or more VUS that could potentially act together to cause the disease, supporting the hypothesis of an oligogenic effect, which has been described before (18).

The oligogenic effect of combinations of low penetrance SNVs in cancer-related genes has been suspected for a long time, including in young patients with breast and lung cancers. In a case-control study of 631 women with breast cancer diagnosed under the age of 53, it was proposed that SNVs in ten

Table IV. Single nucleotide variants identified in the patients.

Patient	Gene	Variant	Effect	Predictors classifying the variant as deleterious	GnomAD frequency	Literature reports	Classification
P1	*CHEK2*	c1100delC (p.Thr367Metfs*15)	Nonsense	CADD	0.17%	Associated with higher risk of breast, prostate and colorectal cancer (12)	LPV
	EME2	c.964C>T (p.Gln322*)	Nonsense	Mutation taster, FATHMM MKL, SIPHY	0.82%	Reported in individual with pancreatic ductal adenocarcinoma at 60 years (13)	VUS
	ATRIP	c.1637T>G (p.Leu546Trp)	Missense	SIFT, PROVEAN, FATHMM MKL, SIPHY	0.00%	-	VUS
P2	*HNF1A*	c.92G>A (p.Gly31Asp)	Missense	Mutation taster, FATHMM, PROVEAN, SIPHY	0.08%	Identified in a female with clear RCC and two chromophobe RCC at age 75 years. No second variant was found (14). Identified in person with MSS CRC at 58 years (15)	VUS
	TSC2	c.5383C>T (p.Arg1795Cys)	Missense	SIFT, POLYPHEN, Mutation Taster, FATHMM, PROVEAN, SIPHY	0.15%	Reported as probably neutral in functional study using 293T cells (16). Reported in individual at high risk for breast and/or ovarian cancer (17)	VUS
P3	*ATM*	c.8988-1G>A	Splicing	SIFT, Mutation Taster, PROVEAN, SIPHY, SPLICEAI AG, SPLICEAI AL, SPLICING ADA	0%	A splicing variant at this position was proven to alter the splice acceptor site (18)	PV
	APC	c.295C>T (p.Arg99Trp)	Missense	SIFT, FATHMM, PROVEAN, FATHMM MKL, SIPHY	0.04%	Detected in a person from a cohort with B-cell neoplasms (19). Identified in one of 40 unrelated patients with familial CRC without classical familial adenomatous polyposis coli (20)	VUS
P4	-	-	-	-	-	-	-
P5	*ERCC2*	c.2260G>C (p.Glu754Gln)	Missense	PROVEAN, SIPHY	0.01%	-	VUS
P6	*RASA2*	c.865T>C (p.Tyr289His)	Missense	SIFT, Mutation Taster, PROVEAN, FATHMM MKL, SIPHY	0%	-	VUS
P7	*RIF1*	c.1475A>G (c.1475A>G)	Missense	SIFT, Mutation Taster, PROVEAN, FATHMM MKL, SIPHY	0%	-	VUS
P8	*CHEK2*	c.434G>A (p.Arg145Gln)	Missense	SIFT, POLYPHEN, Mutation Taster, FATHMM, PROVEAN, FATHMM MKL, SIPHY	0.00%	Reported in a female with breast cancer at 37 years (21). Found in one individual with CRC and in none of the cancer free controls (22). A yeast *in vivo* assay showed that the variant was benign (23)	VUS

Table IV. Continued.

Patient	Gene	Variant	Effect	Predictors classifying the variant as deleterious	GnomAD frequency	Literature reports	Classification
P9	*ATM*	c.2057T>A (p.Leu686His)	Missense	Mutation taster, PROVEAN, FATHMM MKL, SIPHY	0.00%	Reported in a female older than 40 years with breast cancer (25)	VUS
P10	*MUTYH*	c.536A>G (p.Tyr179Cys)	Missense	SIFT, Mutation taster, FATHMM, PROVEAN, FATHMM MKL, SIPHY	0.15%	Observed in homozygous and compound heterozygous state in multiple individuals with MUTYH-Associated Polyposis (10)	PV

CRC, colorectal cancer; LPV, likely pathogenic variant; MSS, microsatellite stable; RCC, renal cell carcinoma; PV, pathogenic variant; VUS, variant of uncertain significance. Reference transcripts: APC NM_000038.6, ATM NM_000051.3, ATRIP NM_130384.2, BRCA1 NM_007294.3, CHEK2 NM_007194.3, EME2 NM_001257370.1, ERCC2 NM_000400.4, HNF1A NM_000545.5, MUTYH NM_012222.2, RASA2 NM_006506.5, RIF1 NM_018151.4, TSC2 NM_000548.5.

genes with known or predicted roles in breast cancer interact to affect a woman's cancer risk in a way unpredictable from single gene effects (19). On the other hand, sets of germline SNVs were identified in young non-smokers with lung adenocarcinoma that underwent WES (20).

The additive effect of multiple genetic events influencing a few biochemical mechanisms was observed in patient 1, where the identified variants were mapped into the cancer-related pathways. The SNVs affected genes of the DNA repair pathways (double-strand break repair and Fanconi anemia: *CHEK2*, *ATRIP*) and cell cycle checkpoints (*EME2*), while the genes involved in the microdeletion were mapped into the cell cycle checkpoints (*BUB1*). The pathogenic variant observed in *CHEK2* could explain each of the cancers observed in the patient separately due to the reports that *CHEK2* can be associated with risk of melanoma (21), seminoma (22), and other cancers (23). However, each of these reported patients showed a narrower spectrum of cancer, therefore the contribution of other pathogenic variants cannot be ruled out to explain multiple cancers in a single patient. The study of this single patient with a very significant number of precocious malignant tumors thus suggests an oligogenic effect. This patient, now healthy at the age of 46, benefits from a regular clinical and imaging surveillance. However, genetic counselling for his 5 children is very difficult; so far, family testing was not proposed as patient 1 had his first diagnosis at the age of 18 (a melanoma), a clinical follow-up from the age of 13 should probably be proposed.

Patient 2 in addition to two SNVs presented a deletion of the four last exons in *MSR1* gene. Germline variants in these gene have been linked to prostate cancer (24) and esophageal carcinoma (25). The importance of this gene in breast cancer and melanoma is yet to be identified. The second CNV was a complete deletion of *APOBEC3B* gene in patient 7. Germline deletions of this gene have been associated with breast cancer risk (26). The CNVs identified in patient 2 and patient 7 were confirmed by nanopore sequencing.

Three of the patients presented microdeletions/microduplications that could alter micro-RNA expression. However, none of the micro-RNA included in the CNVs has been associated with germline risk of cancer.

The other patients from our cohort showed limited numbers of variants or not any identified variant (patient 4). Of course, we cannot exclude mutations in non-coding areas. Other explanations could include epigenetic or environmental factors. Interestingly, there were not any variants identified in patient 4, who presented with a medulloblastoma at an early age, and a bilateral mucinous ovarian borderline tumor later in life, while her monozygotic twin sister never had a tumor, which suggested a non-genetic cause or low penetrance factors that we could not detect.

In five patients, we could analyze the tumors to search for second hits and confirm the involvement of germline variants in tumor suppressor genes. We could not identify such somatic genetic events. However, for further studies, a simultaneous analysis of the germline and tumor DNA should be done, including epigenetic analysis of the tumor DNA.

On the tumors, we investigated the mutational signatures, as they could orient the investigation toward specific oncologic mechanisms, such as DNA repair defects. We could not identify a recurrent signature in the different tumors

from a single patient, except for a defective DNA mismatch repair (MMR) signature observed in the three cancers from patient 6, in which we have not identified any mutation in the MMR genes. Interestingly, several tumors showed a signature indicating exposure to chemotherapy. Although these patients were not previously exposed to chemotherapy nor radiotherapy, exposure to therapeutic or environmental DNA-damaging agents could contribute significantly to cancer risk if there is any constitutional defect in DNA-repair pathways (27).

Our study thus suggests that the simultaneous presence of multiple germline variants can confer a significant cancer risk. We also observed three families with multiple early cancers in patients carrying *BRCA2* and *ATM* mutations (28) and other reports showed similar data (29,30). Moreover, the polygenic risk scores can identify a small population with high cancer risk, as demonstrated for breast cancer (31). Finally, rare recessive conditions could also induce a significant cancer risk as indicated for *MCM9* mutations associated with primary ovarian insufficiency and cancer risk (32). Taken together these studies indicate that: i) investigations should not be limited to single gene studies in patients/families with multiple and/or early cancers; ii) In order to identify all the genetic events that could be associated with cancer genes, SNVs, CNVs, and chromosomal rearrangements including gene fusions should be tested combining different techniques such as NGS and optical genome mapping; iii) further studies are needed to investigate the respective role of genetic and epigenetic events in these patients; iv) the exact role of the observed variants and their cumulative effect should be addressed by functional studies.

The limitations of the study include the small number of participants, the challenges of correlating VUS with the disease and the limited availability of somatic samples. The limited number of participants restricts the generalizability of the findings, and makes impossible a mutation frequency analysis to identify common mutation patterns or recurring PVs. At the moment, it will be not possible to reclassify the VUS into other categories, as the information on the variants is limited, these variants are not recurrent in several patients and consequently there is not enough evidence of pathogenicity to initiate functional validation of the variants. Further studies such as dynamic variant analysis, data integration and bioinformatic analysis can provide insights into the variant role in MPCs, and discover potential biomarkers, signaling pathways, and therapeutic targets. Furthermore, it will be necessary to evaluate the effect of gene-gene, gene-environment and protein-protein interactions, and the role of genetic modifiers and environmental factors in MPCs. Indeed, it will be a real challenge to study the role of oligogenic variants in cancer predisposition. Among possibilities, it could be envisioned to inactivate several genes in cell models and study specific biochemical pathways, controlling for instance cell proliferation, apoptosis or DNA repair mechanisms. In these cell models, and in human tumors studied in parallel, proteomic and transcriptomic studies will evaluate simultaneous loss of gene or protein expression as well as protein-protein interactions. In animal models, one could cross heterozygous or homozygous knock-out animals and evaluate spontaneous or induced cancer development.

Acknowledgements

The CHU Liège is a member of the GENTURIS (GENetic TUmor RISk) European Reference Network.

Funding

This work was supported by Televie (grant no. 7451419F); CHU Liege (grant no. 981481200); Region Wallonne (grant no. 1710180).

Availability of data and materials

The whole exome sequencing data generated in the present study may be found in the SRA database under PRJNA1127072 or at the following URL: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1127072. The chromosomal microarray analysis data generated in the present study may be found in the Gene Expression Omnibus database under accession number GSE271498 or at the following URL: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE271498.

Authors' contributions

VB conceptualized the present study. MF, MM and VB collected and analyzed the data for the present study. MF was in charge of the formal analysis of the study. VB performed the funding acquisition. MF, MM, RT, MA, KD, LHe, MD, KS, NL, AL, JG, LHa and CF performed the investigation. MF, MM, RT, CJ and LP defined the methodology. VB administered the project and acquired the resources. CJ, AL, LP and VB checked and validated the data analysis. MF and VB wrote the original draft. MF, RT, MM, MA, KD, LHe, CF, MD, AL, NL, KS, JG, LHa, LP, CJ and VB reviewed and edited the manuscript. MF, JG and MD confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

This study was approved by the Comité d'Éthique Hospitalo-Facultaire Universitaire de Liège (approval no. 2019/245). All subjects provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Dickie L, Johnson CH, Adams S and Serban N (eds): Solid tumor rules. Effective with Cases Diagnosed 1/1/2018 and Forward. National Cancer, Rockville, MD, 2021. https://seer.cancer.gov/tools/solidtumor/STM.pdf. Accessed March 9, 2023.
2. Report WG: International rules for multiple primary cancers (ICD-0 third edition). Eur J Cancer Prev 14: 307-308, 2005.

3. American Cancer Society: Cancer Facts & Figures 2009. American Cancer Society, Atlanta , GA, 2019. https://www.cancer.org/research/cancer-facts-statistics/all-cancer-facts-figures/cancer-facts-figures-2009.html. Accessed April 6, 2023.
4. Cybulski C, Nazarali S and Narod SA: Multiple primary cancers as a guide to heritability. Int J Cancer 135: 1756-1763, 2014.
5. Chan GHJ, Ong PY, Low JJH, Kong HL, Ow SGW, Tan DSP, Lim YW, Lim SE and Lee SC: Clinical genetic testing outcome with multi-gene panel in Asian patients with multiple primary cancers. Oncotarget 9: 30649-30660, 2018.
6. Harvengt J, Lumaka A, Fasquelle C, Caberg JH, Mastouri M, Janssen A, Palmeira L and Bours V: HIDEA syndrome: A new case report highlighting similarities with ROHHAD syndrome. Front Genet 14: 1137767, 2023.
7. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, *et al*: Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American college of medical genetics and genomics and the association for molecular pathology. Genet Med 17: 405-424, 2015.
8. Backenroth D, Homsy J, Murillo LR, Glessner J, Lin E, Brueckner M, Lifton R, Goldmuntz E, Chung WK and Shen Y: CANOES: Detecting rare copy number variants from whole exome sequencing data. Nucleic Acids Res 42: e97, 2014.
9. Riggs ER, Andersen EF, Cherry AM, Kantarci S, Kearney H, Patel A, Raca G, Ritter DI, South ST, Thorland EC, *et al*: Technical standards for the interpretation and reporting of constitutional copy number variants: A joint consensus recommendation of the American college of medical genetics and genomics (ACMG) and the clinical genome resource (ClinGen). Genet Med 22: 245-257, 2020.
10. Li MM, Datto M, Duncavage EJ, Kulkarni S, Lindeman NI, Roy S, Tsimberidou AM, Vnencak-Jones CL, Wolff DJ, Younes A and Nikiforova MN: Standards and guidelines for the interpretation and reporting of sequence variants in cancer. J Mol Diagn 19: 4-23, 2017.
11. Blokzijl F, Janssen R, van Boxtel R and Cuppen E: MutationalPatterns: Comprehensive genome-wide analysis of mutational processes. Genome Med 10: 33, 2018.
12. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, Bignell GR, Bolli N, Borg A, Børresen-Dale AL, *et al*: Signatures of mutational processes in human cancer. Nature 500: 415-421, 2013.
13. Nielsen M, Joerink-van de Beld MC, Jones N, Vogt S, Tops CM, Vasen HFA, Sampson JR, Aretz S and Hes FJ: Analysis of MUTYH genotypes and colorectal phenotypes in patients with MUTYH-associated polyposis. Gastroenterology 136: 471-476, 2009.
14. Barreiro RAS, Sabbaga J, Rossi BM, Achatz MIW, Bettoni F, Camargo AA, Asprino PF and Galante PAF: Monoallelic deleterious MUTYH germline variants as a driver for tumorigenesis. J Pathol 256: 214-222, 2022.
15. Abou Tayoun AN, Pesaran T, DiStefano MT, Oza A, Rehm HL, Biesecker LG and Harrison SM; ClinGen Sequence Variant Interpretation Working Group (ClinGen SVI): Recommendations for interpreting the loss of function PVS1 ACMG/AMP variant criterion. Hum Mutat 39: 1517-1524, 2018.
16. Whitworth J, Smith PS, Martin JE, West H, Luchetti A, Rodger F, Clark G, Carss K, Stephens J, Stirrups K, *et al*: Comprehensive cancer-predisposition gene testing in an adult multiple primary tumor series shows a broad range of deleterious variants and atypical tumor phenotypes. Am J Hum Genet 103: 3-18, 2018.
17. Liu YL, Cadoo KA, Mukherjee S, Khurram A, Tkachuk K, Kemel Y, Maio A, Belhadj S, Carlo MI, Latham A, *et al*: Multiple primary cancers in patients undergoing tumor-normal sequencing define novel associations. Cancer Epidemiol Biomarkers Prev 31: 362-371, 2022.
18. McGuigan A, Whitworth J, Andreou A, Hearn T, Tischkowitz M and Maher ER: Multilocus inherited neoplasia allele syndrome (MINAS): An update. Eur J Hum Genet 30: 265-270, 2022.
19. Aston CE, Ralph DA, Lalo DP, Manjeshwar S, Gramling BA, DeFreese DC, West AD, Branam DE, Thompson LF, Craft MA, *et al*: Oligogenic combinations associated with breast cancer risk in women under 53 years of age. Hum Genet 116: 208-221, 2005.
20. Renieri A, Mencarelli MA, Cetta F, Baldassarri M, Mari F, Furini S, Piu P, Ariani F, Dragani TA and Frullanti E: Oligogenic germline mutations identified in early non-smokers lung adenocarcinoma patients. Lung Cancer 85: 168-1674, 2014.
21. Weischer M, Heerfordt IM, Bojesen SE, Eigentler T, Garbe C, Röcken M, Hölmich LR, Schmidt H, Klyver H, Bastholt L and Nordestgaard BG: CHEK2*1100delC and risk of malignant melanoma: Danish and German studies and meta-analysis. J Invest Dermatol 132: 299-303, 2012.
22. AlDubayan SH, Pyle LC, Gamulin M, Kulis T, Moore ND, Taylor-Weiner A, Hamid AA, Reardon B, Wubbenhorst B, Godse R, *et al*: Association of inherited pathogenic variants in checkpoint kinase 2 (CHEK2) with susceptibility to testicular germ cell tumors. JAMA Oncol 5: 514-522, 2019.
23. Stolarova L, Kleiblova P, Janatova M, Soukupova J, Zemankova P, Macurek L and Kleibl Z: CHEK2 germline variants in cancer predisposition: Stalemate rather than checkmate. Cells 9: 2675, 2020.
24. Xu J, Zheng SL, Komiya A, Mychaleckyj JC, Isaacs SD, Hu JJ, Sterling D, Lange EM, Hawkins GA, Turner A, *et al*: Germline mutations and sequence variants of the macrophage scavenger receptor 1 gene are associated with prostate cancer risk. Nat Genet 32: 321-325, 2002.
25. Orloff M, Peterson C, He X, Ganapathi S, Heald B, Yang YR, Bebek G, Romigh T, Song JH, Wu W, *et al*: Germline mutations in MSR1, ASCC1, and CTHRC1 in patients with barrett esophagus and esophageal adenocarcinoma. JAMA 306: 410-419, 2011.
26. Wen WX, Soo JS, Kwan PY, Hong E, Khang TF, Mariapun S, Lee CS, Hasan SN, Rajadurai P, Yip CH, *et al*: Germline APOBEC3B deletion is associated with breast cancer risk in an Asian multi-ethnic cohort and with immune cell presentation. Breast Cancer Res 18: 56, 2016.
27. Vega-Stromberg T: Chemotherapy-induced secondary malignancies. J Infus Nurs 26: 353, 2003.
28. Freire Chadrina M, Martin M and Segers K: Sepulchre. Coinheritance of pathogenic variants in ATM and BRCA2 in families with multiple cancers: A case series (Internet). 2022 (cited 2023 Feb 23).
29. Vietri MT, Caliendo G, D'Elia G, Resse M, Casamassimi A, Minucci PB, Dello Ioio C, Cioffi M and Molinari AM: Five Italian families with two mutations in BRCA genes. Genes (Basel) 11: 1451, 2020.
30. Huang W, Bian J, Qian X, Shao L, Li H, Zhang L and Wang L: Case report: Coinheritance of germline mutations in APC and BRCA1 in colorectal cancer. Front Oncol 11: 658389, 2021.
31. Borde J, Ernst C, Wappenschmidt B, Niederacher D, Weber-Lassalle K, Schmidt G, Hauke J, Quante AS, Weber-Lassalle N, Horváth J, *et al*: Performance of breast cancer polygenic risk scores in 760 female CHEK2 germline mutation carriers. J Natl Cancer Inst 113: 893-899, 2020.
32. Potorac I, Laterre M, Malaise O, Nechifor V, Fasquelle C, Colleye O, Detrembleur N, Verdin H, Symoens S, De Baere E, *et al* : The role of MCM9 in the etiology of sertoli cell-only syndrome and premature ovarian insufficiency. J Clin Med 12: 990, 2023.