Follow-up System for Childhood Cancer Survivors Via Germline Clinical Sequencing

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Background : A second malignant neoplasm (SMN) has the greatest impact on the prognosis of childhood cancer survivors (CCSs). Although germline abnormalities in cancer predisposition genes have been reported as a cause of SMN in CCSs, the genetic background is not considered for SMN surveillance in the follow-up guidelines. This study aimed to present an SMN surveillance system for CCSs using germline cancer predisposition genes and evaluate their efficacy. We also aimed to elucidate the psychological impact of a surveillance system on CCSs and their guardians.

Methods : CCSs who visited the long-term follow-up clinic at Shinshu University Hospital were recruited. They underwent next-generation sequencing-based germline genetic investigation using a custom panel including 165 cancer predisposition genes and a multiplex ligation-dependent probe amplification method for *TP53*. Based on the molecular findings, appropriate SMN surveillance was proposed. A questionnaire-based survey was conducted to comprehend the thoughts of CCSs and/or their guardians regarding SMN, clinical sequencing, and SMN surveillance.

Results : As of March 2021, 16 CCSs, mostly with leukemia as a primary cancer, participated in this study. No pathogenic or likely pathogenic variants were detected in any of the participants. Variants of uncertain significance were found in four CCSs showing increased anxiety.

Conclusions : This study could not show the efficacy of an SMN surveillance system for CCSs, because no pathogenic or likely pathogenic variants were detected. Further evaluation, including more CCSs with a wider spectrum of cancers, would be necessary to evaluate this system. Genetic counseling might require careful anticipatory guidance for clinical sequencing and follow-up services. *Shinshu Med J 70 : 157—167, 2022*

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Key words : cancer predisposition genes, childhood cancer survivor, clinical sequencing, follow-up system, second malignant neoplasm

I Introduction

The improvement in the cure rate of pediatric cancer in recent years has led to an increase in childhood cancer survivors (CCSs)[1)2)]. Consequently, late effects in CCSs have become a serious problem[3)]. A second

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malignant neoplasm (SMN) has the greatest impact on long-term life prognosis among the late effects[4]. Therefore, early detection and early stage treatment of CCSs with SMN is very important to improve their long-term life prognosis[5].

Anticancer drugs and/or radiation therapy are well-known causes of SMN. Additionally, germline abnormalities in cancer predisposition genes in CCSs have also been reported as a cause of SMN in recent years[6]. CCSs have been shown to have a higher probability of carrying germline abnormalities in cancer predisposition genes than the general population[6)7)]. Hence, the assessment of germline abnormalities in cancer predisposition genes for CCSs could be useful for the stratification of the risk for development of SMN. However, in the current follow-up guidelines for CCSs, the genetic background is considered only in some SMN screening systems, such as breast cancer (BRCA 1/2), and the stratification of SMN risk is mainly based on the history of anticancer therapies[8].

Therefore, we introduced a surveillance system using clinical sequencing of germline cancer predisposition genes for CCSs to assess the risk of developing SMN in a long-term follow-up (LTFU) clinic at Shinshu University Hospital. The purpose of this study was to present the clinical and molecular findings in CCSs, application of SMN surveillance, and discuss the thoughts of the participants obtained through this follow-up system. The study also aimed to evaluate the efficacy of the follow-up system.

Ⅱ Methods

A Participants

An outline of this study is presented in **Fig. 1**. The participants were CCSs who were recruited from the LTFU clinic at Shinshu University Hospital. The LTFU clinic was established in 2014 and included approximately 140 CCSs as of October 2020, some of whom were introduced from other hospitals after the completion of their cancer therapy. This study was approved by the Ethics Committee of Shinshu University School of Medicine in January 2019 (approval number : 633) and was initiated in August 2019.

We informed the CCSs and/or their guardians of this study at the LTFU Clinic of the Department of Pediatrics in Shinshu University Hospital. Upon agreeing to participate in the study, CCSs and/or their guardians were referred to the Center for Medical Genetics for genetic counseling for clinical sequencing provided by a clinical geneticist (TK), a pediatric oncologist (TW), and certified genetic counselors. Informed consent was obtained from adult CCSs and guardians of minor CCSs (younger than 20 years old). As of March 2021, 16 CCSs participated in the study.

B Clinical sequencing

After obtaining consent from the participants and/or their guardians, 5 mL of peripheral blood was collected from CCSs with no history of allogeneic hematopoietic stem cell transplantation (allo-HSCT). In contrast, both nails and hair were collected from CCSs with a history of allo-HSCT. To extract genomic DNA from the samples, QIAcube (Qiagen, Hilden, Germany) was used for peripheral blood, and ISOHAIR (NIPPON GENE, Tokyo, Japan) was used for nails and hair. All exons and the flanking sequences of 165 cancer predisposition genes **(Table 1)** were analyzed using a next-generation sequencer, Ion GeneStudio S5 (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. These 165 cancer predisposition genes, selected from literature reviews and National Comprehensive Cancer Network (NCCN) guidelines, have been reported to cause cancers associated with germline abnormalities[6)9)-16)].

Sequencing data were mapped to human genome hg19 using Torrent Suite software (Thermo Fisher Scientific), and single nucleotide variants and small insertions/deletions were detected from the mapped data using the Torrent Variant Caller plug-in. Detected variants were annotated using SnpEff[17] and SnpSift using the processed vcf file of the Genome Aggregation Database (gnomAD) version 2.1.1[18] and Human Genetic Variation Database version 2.3[19]. Missense variants were analyzed with dbNSFP3.4c, and splice site alterations were analyzed using dbscSNV1.1. Integrative Genomics Viewer (Broad Institute, Cambridge, MA, USA) was used to visualize the read alignments and sequencing errors.

In addition, copy number variants were analyzed

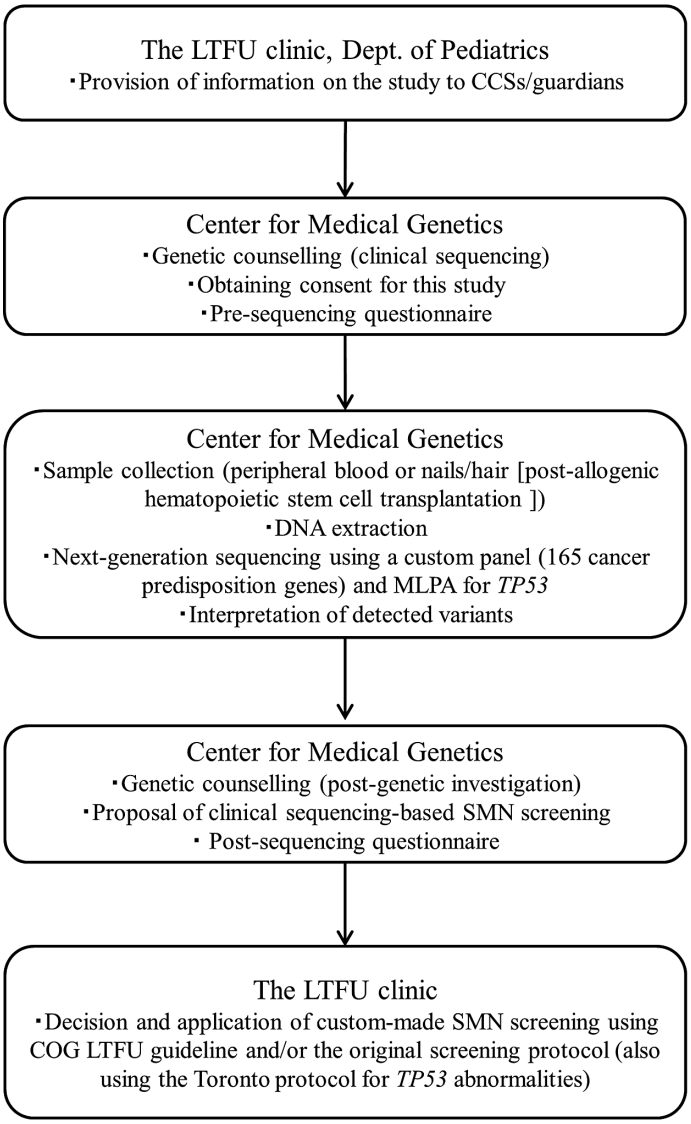


Fig. 1 Outline of this study
COG, Children's Oncology Group ; LTFU, long-term follow-up ; SMN, second malignant neoplasm

Table 1 List of 165 cancer predisposition genes analyzed in this study

ABCB11, ACD, ALK, ANKRD26, APC, ATM, AXIN2, BAP1, BLM, BMPR1A, BRAF, BRCA1, BRCA2, BRIP1, BUB1B, CBL, CDC73, CDH1, CDK4, CDKN1B, CDKN1C, CDKN2A, CEBPA, CHEK2, COL7A1, CTC1, CYLD, DDB2, DDX41, DICER1, DIS3L2, DKC1, DOCK8, EGFR, ELANE, EPCAM, ERCC1, ERCC2, ERCC3, ERCC5, ETV6, EXT1, EXT2, FAH, FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCL, FANCM, FANCQ, FANCR, FANCT, FH, FLCN, GATA2, GBA, GFI1, GJB2, GPC3, GREM1, HAX1, HFE, HMBS, HRAS, IKZF1, ITK, KIT, KRAS, MAP2K1, MAP2K2, MAX, MEN1, MET, MLH1, MPL, MSH2, MSH3, MSH6, MTAP, MUTYH, NBN, NF1, NF2, NHP2, NOP10, NRAS, NTHL1, PALB2, PAX5, PDGFRA, PHOX2B, PMS2, POLD1, POLE, POLH, PRKAR1A, PRSS1, PTCH1, PTEN, PTPN11, RAD51C, RAD51D, RAF1, RB1, RBBP6, RECQL4, RET, RHBDF2, RMRP, RPL5, RPL11, RPL35A, RPS10, RPS17, RPS19, RPS24, RPS26, RTEL1, RUNX1, SAMD9, SAMD9L, SBDS, SDHA, SDHAF2, SDHB, SDHC, SDHD, SERPINA1, SH2B3, SH2D1A, SHOC2, SLC25A13, SLX4, SMAD4, SMARCA4, SMARCB1, SMARCE1, SOS1, SRY, STAT3, STK11, SUFU, TERC, TERT, TGFBR1, TINF2, TMEM127, TNFRSF6, TP53, TRIM37, TSC1, TSC2, UROD, VHL, WAS, WRAP53, WRN, WT1, XPA, XPC

using the multiplex ligation-dependent probe amplification method for *TP53*, the most common gene related to leukemia and pediatric cancers, using Applied Biosystems VRTi Dx and Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's protocol.

Detected variants were assessed through Human Gene Mutation Database professional 2020.1 (Qiagen) and ClinVar[20]. If not registered, they were interpreted according to the 2015 American College of Medical Genetics and Genomics or the Association for Molecular Pathology guidelines[21] by a clinical geneticist (TK), pediatric oncologist (TW), and molecular geneticist (TY). Genetic counseling was provided to CCSs and/or their guardians by presenting the results of clinical sequencing and relevant surveillance plans.

C Surveillance for SMN

When pathogenic or likely pathogenic variants were detected in *TP53*, surveillance for SMN was proposed according to the Toronto protocol[5] in combination with the guidelines of the Children's Oncology Group (COG) LTFU program[8].

When pathogenic or likely pathogenic variants in other genes, for which surveillance methods were specified in the NCCN guidelines[22], American Association for Cancer Research-Childhood Cancer Predisposition Workshop review articles[23)-39)], and/or GeneReviews[40], SMN surveillance was proposed according to the COG LTFU guidelines. In contrast, when pathogenic or likely pathogenic variants were detected in genes for which no guidelines were established, SMN surveillance was proposed based on previous reports in combination with COG LTFU guidelines.

D Questionnaire-based survey

To comprehend the thoughts of CCSs and/or their guardians regarding SMN, clinical sequencing, and SMN surveillance, a questionnaire-based survey was conducted. The questionnaire consisted of six questions, each of which had five choices (strongly disagree, disagree a little, neither agree nor disagree, agree a little, and strongly agree) **(Table 2)**. The same survey was conducted before and after clinical sequencing. To assess differences in their thoughts between the two surveys, a paired *t*-test was performed using the GraphPad Prism software package (version 9.2 ; GraphPad Software, San Diego, CA, USA).

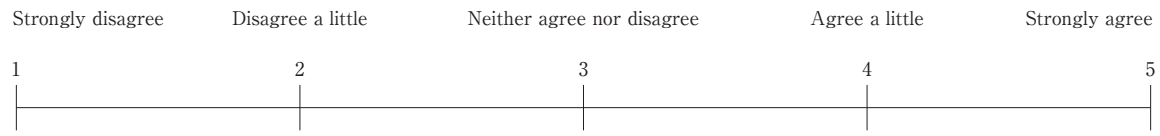
Ⅲ Results

As of March 2021, 37 CCSs were informed of this study, and 16 of them wished to participate. The clinical and molecular findings of the participants are presented in **Table 3**. Types of childhood cancer included acute lymphoblastic leukemia (ALL, n = 9), acute myeloid leukemia (AML, n = 2), neuroblastoma (n = 2), myeloid/NK cell precursor acute leukemia (n = 1), juvenile myelomonocytic leukemia (n = 1), and Wilms tumor (n = 1). The median age of the CCSs undergoing clinical sequencing was 22 years (range, 15-42 years), six of whom were minors. The male-to-female sex ratio was 1 : 1. Four CCSs had a history of SMN. No pathogenic variants corresponding to likely pathogenic or pathogenic variants were detected in any of the participants. Twelve variants of uncertain significance (VUSs) were detected in 10 participants. Most VUSs (eight of 12) were missense variants.

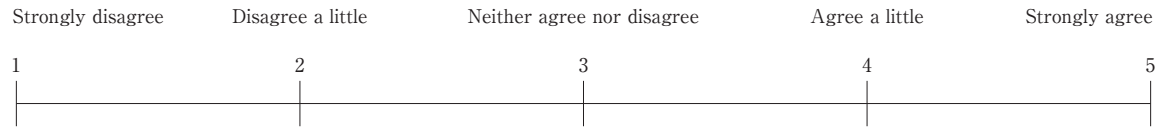
Since Patient 1 had four cancers (ALL, rectal cancer, ovarian cancer, and breast cancer) and a family history of gastric cancer, she was supposed to have a germline pathogenic variant of some cancer predisposition genes. Contrary to our expectations, only the VUSs of *SAMD9L* and *POLD1* genes were detected. No specific SMN surveillance has been proposed to date. Patient 10 developed three tumors (neuroblastoma, cervical cancer, and meningioma), but only a VUS of the *MPL* gene was detected. No specific SMN surveillance has been proposed to date. Patient 13 developed ALL and meningioma, but no pathogenic variants were detected. In Patient 16, who developed AML, a heterozygous variant was detected in the *MUTYH* gene. The variant was interpreted as a VUS, according to ClinVar®[20], in which the variant was registered as likely benign in three cases, VUS in five cases, likely pathogenic in three cases, and pathogenic in one case. *MUTYH*-related polyposis is an autosomal recessive disease associated with an increased risk of colorectal cancer (CRC) ; however,

Table 2　The questionnaire used in this study

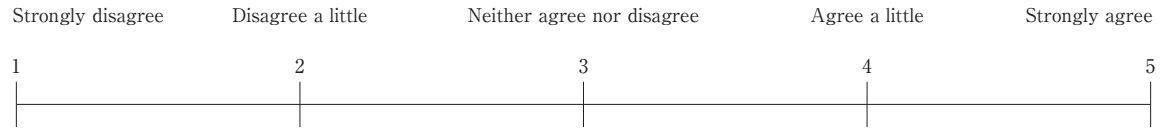
Q1. Are you worried about your child developing a second malignant neoplasm (SMN) in the future?



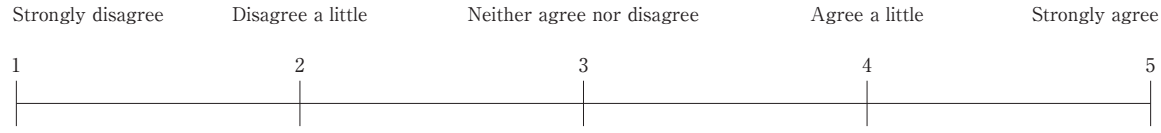
Q2. Do you think that this clinical sequencing of cancer predisposition genes is useful for yourself (or your child)?



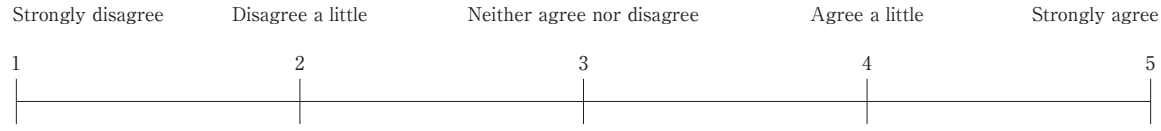
Q3. Do you want to tell the results of this clinical sequencing of cancer predisposition genes to your family?



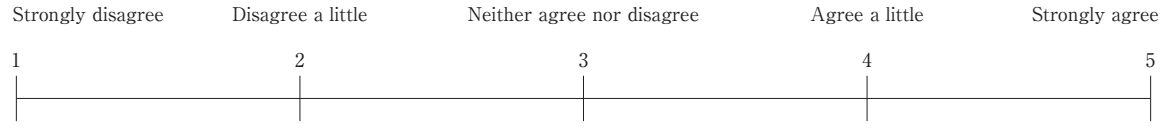
Q4. Do you want to receive regular follow-up examinations of late effects (including SMN) in the future?



Q5. Do you want to receive periodic follow-up by a clinical geneticist and/or certified genetic counselor?



Q6. Do you want to know about late effects apart from the SMN?



the risk of CRC and extraintestinal cancer in individuals with pathogenic variants in *MUTYH* is unclear[41]. Since Patient 16 was treated with anticancer agents and underwent allo-HSCT using total body irradiation, he was considered to have a relatively high risk of SMN. Therefore, we proposed the option of SMN surveillance in this case. No specific or further SMN surveillance was proposed for other patients with VUSs.

We also conducted a questionnaire-based survey before and after clinical sequencing of the CCSs or guardians who participated in this study **(Table 2)**. **Table 4** summarizes the results of the questionnaire-based survey. No significant change in scores was observed in any of the questions before and after clinical sequencing. For the first question, which focused on anxiety about SMN, the results were unchanged before and after clinical sequencing in seven CCSs. However, anxiety increased in four CCSs, and decreased in five CCSs. All four patients with in-

Table 3　Clinical and molecular findings in 16 participants

Patient no.	Age at clinical sequencing (years)	Sex	Primary disease	Age at onset of primary disease (years)	Second tumor (age, years)	Family history of malignancy	Detected variant(s)		
							P	LP	VUS
1	42	F	ALL	15	Rectal cancer (30) Ovarian cancer (30) Breast cancer (42)	Father : gastric cancer Paternal grandfather : gastric cancer	–	–	*SAMD9L* (NM_152703.3 : c.2256G＞A) : missense variant *POLD1* (NM_002691.3 : c.1138-7C＞A) : splice-site variant
2	22	M	ALL	14	Colorectal polyps (20, 22)	Maternal grandfather : colorectal cancer	–	–	*ATM* (NM_000051.3 : c.323C＞G) : missense variant *BRIP1* (NM_032043.2 : c.2830C＞G) : missense variant
3	27	F	Myeloid/NK cell precursor acute leukemia	10	–	Paternal grandmother : cancer	–	–	*POLE* (NM_006231.3 : c.76A＞G) : missense variant
4	37	M	ALL	12	–	Maternal grandmother : cancer	–	–	–
5	16	M	ALL	4	–	–	–	–	*CDK4* (NM_000075.3 : c.887A＞G) : missense variant
6	24	M	ALL	13	–	–	–	–	–
7	15	F	ALL	13	–	Maternal grandfather : cholangiocarcinoma Maternal grandmother : breast cancer	–	–	*BRCA1* (NM_007294.3 : c.626C＞T) : missense variant
8	16	F	JMML	15	–	Maternal grandmother : uterine cancer	–	–	–
9	19	M	ALL	14	–	Mother : bilateral breast cancer	–	–	*MSH6* (NM_000179.2 : c.3772C＞G) : missense variant
10	32	F	NB	0	Cervical cancer (20) Meningioma (31)	Maternal grandfather : prostate cancer	–	–	*MPL* (NM_005373.2 : c.853G＞T) : missense and/or splice-site variant
11	22	M	AML	0	–	Paternal grandmother : malignant lymphoma	–	–	*SAMD9L* (NM_152703.3 : c.2357C＞G) : missense variant
12	34	F	NB	6	–	Mother : breast cancer Paternal grandmother : kidney cancer	–	–	–
13	30	M	ALL	5	Meningioma (22)	Paternal grandfather : gastric cancer, lung cancer Maternal grandfather : gastric cancer	–	–	–
14	20	F	WT	5	–	–	–	–	–
15	16	F	ALL	3	–	–	–	–	*GJB2* (NM_004004.5 : c.508_511dup) : frameshift variant
16	16	M	AML	9	–	Maternal grandfather : lung cancer	–	–	*MUTYH* (NM_001128425.2 : c.934-2A＞G) : splice-site variant

ALL, acute lymphoblastic leukemia ; AML, acute myeloid leukemia ; F, female ; JMML, juvenile myelomonocytic leukemia ; LP, likely pathogenic ; M, male ; NK, natural killer ; P, pathogenic ; VUS, variant of uncertain significant ; WT, Wilms tumor

Table 4 Responses of childhood cancer survivors or their guardians to the questionnaires regarding a novel follow-up system

Patient no.	Q1		Q2		Q3		Q4		Q5		Q6	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1	5	5	5	5	4	4	5	5	5	5	5	5
2	2	2	5	5	5	5	5	5	5	5	4	5
3	3	4	5	4	4	5	5	5	4	5	5	5
4	5	4	4	5	5	5	5	5	4	5	4	4
5	4	5	5	5	5	5	4	4	3	4	4	4
6	4	4	5	5	4	4	5	5	3	3	4	4
7	1	1	5	5	5	5	5	5	5	5	5	4
8	4	2	5	5	5	5	5	5	5	4	5	5
9	3	4	5	5	4	5	4	5	4	5	4	4
10	5	3	5	5	5	5	5	5	5	5	5	5
11	3	4	4	5	4	4	4	5	4	4	4	4
12	4	3	4	4	4	4	4	4	2	4	4	4
13	5	3	5	4	5	5	5	5	5	5	5	5
14	2	2	4	4	4	4	5	5	3	3	4	4
15	4	4	4	4	4	4	4	4	4	3	4	4
16	4	4	5	5	5	5	5	5	3	3	4	4
Mean	3.6	3.4	4.7	4.7	4.5	4.6	4.7	4.8	4.0	4.3	4.4	4.4
p-value	0.362		1.000		0.164		0.164		0.216		1.000	

creased anxiety were CCSs who were found to have VUS.

Ⅳ Discussion

In this study, we described the preliminary data of an originally established SMN surveillance system for CCSs, considering the results of clinical sequencing for germline cancer predisposition genes. The purpose of this system is to detect SMNs in CCSs at an early stage and consequently improve their long-term prognosis. A distinctive feature of this system is the introduction of a germline genetic investigation, as a clinical basis, into a previously established LTFU clinic in the Department of Pediatrics in our hospital. There have been several reports regarding the detection of pathological variants of cancer predisposition genes in CCSs using the gene panel[6)42)43)], but there has been no report of an SMN follow-up system routinely linking clinical sequencing for germline cancer predisposition genes.

No pathogenic or likely pathogenic variants in the 165 germline cancer predisposition genes were detected in this study. The prevalence of pathogenic variants ranged from 5.8 % to 11.5 %[6)42)43)] according to previous reports on germline abnormalities in cancer predisposition genes in CCSs. Possible causes for detecting no pathogenic or likely pathogenic variants in this study are estimated as follows : First, the number of CCSs who underwent clinical sequencing was small. Second, the types of primary cancer were biased, as 13 of 16 participants in the current study developed acute leukemias as primary cancer ; CCSs who had Li-Fraumeni syndrome-related solid tumors (e.g., adrenocortical cancer, osteosarcoma, rhabdomyosarcoma) as primary cancer and were likely to have pathogenic variants in *TP53*[6)] were not included in this study.

Four participants (25 %) developed secondary tumors at the time of participation in this study. Patient 1 had four malignant tumors by the age of 42 and met

the Chompret criteria[44] because a relative developed young-onset cancer. However, no pathogenic variants, including copy number abnormalities in *TP53*, were detected. She received both chemotherapy and allo-HSCT after a 12-Gy total body irradiation-based conditioning regimen for ALL. Chemotherapy at onset consisted of cyclophosphamide, etoposide, pirarubicin, and mitoxantrone, which are at high risk for SMN. In addition, allo-HSCT at the time of relapse may be associated with SMN.

The risk of CRC in individuals heterozygous for a germline *MUTYH* pathogenic variant was slightly increased in large population-based and family-based studies, while the frequency of colonic and upper gastrointestinal polyps did not increase in 62 heterozygotes for *MUTYH*[41]. Although a slightly increased cumulative risk for *MUTYH* heterozygotes for gastric, hepatobiliary, endometrial, and breast cancers has been reported, other case-control studies did not find an association between *MUTYH* heterozygosity and risk for breast cancer or hepatocellular carcinoma[41]. Based on the history of treatment and pathogenicity of the variant, the patient was considered to have a relatively high risk of SMN and an SMN surveillance was proposed as a precautionary measure.

A questionnaire-based survey was conducted to comprehend the thoughts of CCSs and/or their guardians regarding SMN, clinical sequencing, and SMN surveillance. Anxiety about SMN did not change significantly before and after clinical sequencing, but all four CCSs who were found to have VUS showed increased anxiety. This result suggests that the presence or uncertainty of variants in cancer predisposition genes could increase anxiety about SMN for CCSs and their families, even after genetic counseling. Genetic counseling in the current system would require more careful anticipatory guidance for this clinical sequencing (e.g., low detection rates, possibilities of VUS) as well as follow-up services, including variant interpretation and psychological aspects.

During the study period, the current clinical sequencing-based surveillance system was proposed to 37 CCSs and their families in the LTFU clinic, and 16 of them wished to participate in this study and were recruited. In this system, the clinical sequencing and relevant genetic counseling costs approximately 53,000 JPY (about $ 485), which is not covered by the health insurance system in Japan. We were concerned that CCSs and their parents might be reluctant to receive genetic services at their own expense because most of the costs of treatment for specific pediatric chronic diseases, including cancer, are covered by the health insurance system. Low estimated detection rates for pathogenic or likely pathogenic variants and possible anxieties through the risk of SMN and uncovering hereditary cancer predisposing syndromes could have been negative factors for participation in this study. However, the fact that 16 CCSs (43 %) participated in this study suggests that a certain proportion of CCSs and their guardians would potentially like to know the risk of developing SMN. A further questionnaire-based survey regarding participation in this study to the remaining CCSs who did not accept the proposal might clarify the causes of their non-participation.

This study has some limitations. First, the number of participants is small, and experiences of actionable pathogenic variants have been lacking, which would be required for discussing the efficacy of SMN surveillance in this system. Second, the types of primary cancer were biased, as 13 of 16 participants in the current study developed acute leukemias as primary cancer. Third, selection of the target genes (165 cancer predisposition genes) might not have been sufficient. It is presumed that more cancer predisposition genes will be newly discovered in the future, and hence, it would be necessary to update the surveillance guidelines as appropriate. Fourth, the estimated pathogenicity of the detected variants in these cancer predisposition genes could change. Therefore, it would be useful to estimate the pathogenicity of detected variants on a regular basis (e.g., searching the ClinVar® website at the time of the annual LTFU clinic). Fifth, specific interpretation of the detected variants may be difficult (e.g., a heterozygous variant in *MUTYH* might or might not be a risk for CRC or extraintestinal cancer depending on the relevant population or the study design).

In summary, our novel follow-up system for CCSs is presented, comprising germline clinical sequencing of 165 cancer predisposition genes and relevant SMN surveillance. No pathogenic or likely pathogenic variants were detected among the 16 participants, who mainly developed leukemia as a primary cancer, and currently the efficacy of an SMN surveillance system for CCSs could not be shown. The presence or uncertainty of variants in cancer predisposition genes could increase anxiety about SMN in CCSs and their families. Further evaluation, including more CCSs with a wider spectrum of cancers, would be necessary to evaluate this system. Genetic counseling might require careful anticipatory guidance for clinical sequencing and follow-up services, including variant interpretation and psychological aspects.

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Disclosure

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Author Contribution

TW, DM, YN, and TK designed the study ; TY performed molecular investigation and interpreted the molecular data with TW and TK ; KH, DM, EO, HM, SS, MT, and YN recruited participants and collected clinical information ; TW and KH combined and interpreted the whole data (molecular and clinical investigation ; questionnaire-based survey) and wrote the manuscript ; all authors read and approved the final manuscript.

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