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Research Article

Mutational Analysis of Colorectal Adenomatous Polyps in Childhood Cancer Survivors Treated with Radiation Therapy Using Targeted Next Generation Sequencing

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Abstract

Background: Childhood cancer survivors treated with radiation therapy (RT) are at an increased risk of subsequent precancerous and cancerous lesions, including the risk of early invasive colorectal carcinoma (CRC). It is well-established that radiation-associated CRC undergo a preinvasive dysplastic stage where they can be detected and managed by early colonoscopy. Molecular profiling of radiation-associated polyps using targeted Next Generation Sequencing (NGS) is highly sensitive and specific and may aid in unraveling their molecular composition. In this study, we aim to analyze and molecularly characterize colorectal adenomatous polyps in young cancer survivors treated with RT using a targeted NGS panel, as well as correlate the genetic findings with their histomorphology.

Design: We extracted DNA scrolls from 18 polyps of different histomorphologies which we acquired from 17 patients who received RT for childhood cancers. The median age is 35 at the time of colonoscopy. The NGS panel comprises more than 146 hotspots in more than 30 genes including: BRAF, KRAS, TP53, PIK3CA, PTEN and CTNNB1 etc. Four of the polyps had histopathologic diagnoses of sessile serrated lesion (SSL) (22.2%), while the remaining 14 polyps were either tubular adenomas (TA), tubulovillous adenomas (TVA) or TA with submucosal invasion (77.7%). High-risk features were defined as size of 10 mm or greater, three or more adenomas, tubulovillous or villous histology or adenomas with high-grade dysplasia.

Results: Five polyps elucidated hotspot mutations at cancer-associated genes (27.7%), while the remaining 13 polyps were negative for the mutations tested (72.3%). Of the 5 mutated polyps, 2 polyps harbored substitution mutations in the KRAS gene at exon 2. The two polyps were non-serrated and showed high-risk histological features. Additionally, two other polyps showed substitution mutations in the BRAF gene at codon V600E, with both polyps showing histological features of SSL. The last mutated polyp had a histological diagnosis of TA with submucosal invasion and elucidated a substitution mutation at the TP53 gene.

Conclusion: This retrospective study is one of the first to molecularly characterize colorectal adenomatous polyps in childhood cancer survivors treated with radiation therapy using Next Generation Sequencing. Our molecular analysis demonstrate that radiation-induced polyps harbor similar genetic changes to dysplastic adenomatous polyps in the average-risk population. We postulate that radiation-associated polyps follow the conventional 'adenoma-carcinoma' pathway or the alternative 'serrated' sequence, making them amenable to detection and management by early colonoscopy. Furthermore, our NGS panel failed to detect novel driving mutations in cancer-associated genes. More comprehensive molecular analysis of these polyps is required to understand their molecular composition and to aid in identifying actionable genetic alterations.

Keywords: *Civil Law Relations; Civil Law Methodology; Probability; Civil Law Movement; Civilistic Categories***Introduction**

Introduction

Childhood cancer survivors treated with radiation therapy (RT) are at an increased risk of subsequent premalignant and malignant lesions including early invasive solid cancers [1]. These neoplasms usually arise at- or near the edge of the radiation field with a risk of up to 6-fold compared to the general population [1,2]. Most of these neoplasms involve the genitourinary system, gastrointestinal system and head and neck area [3,4].

The risk of subsequent GI cancers compared the general population range from 3.2 to 9.7-fold and is proportionate to the radiation dose [5,6]. Colorectal carcinoma (CRC) risk is especially high for patients who were exposed to childhood abdominal/pelvic radiation therapy, as demonstrated by Henderson et al in 2012 [7]. It is suggested that CRC in this patient population is likely to develop from intermediate precursors (adenomatous polyps), as evident by recent studies demonstrating increased prevalence of premalignant colorectal adenomatous polyps in patients aged 50 years or less who received RT for childhood cancers [8,9]. No well-established screening guidelines are available for childhood radiation survivors, nevertheless, it has been proposed that screening colonoscopy is initiated at 10 years after receipt of radiation therapy in an attempt to detect and treat adenomatous polyps at their preinvasive stage and to prevent progression to carcinoma [9].

The molecular profiles of radiation-associated polyps in young childhood cancer survivors is left largely unexplored and our purpose for this study is to detect novel driver or associated mutations which could be implicated in early detection and management. Next generation sequencing offers a highly sensitive and specific method of multiplexing gene sequencing with relative ease and with increasing application in clinical research.

Aim of the Study

In this study, we aim to analyze and molecularly characterize colorectal adenomatous polyps in young cancer survivors treated with RT using NGS and correlate the genetic findings with their histomorphology.

Methods

The study had research ethics approval by the institutional review boards (UBC Ethics #H17-02665; VCH #V17-02665). Childhood cancer survivors who underwent a complete colonoscopy between January 2006 and May 2018 were identified from the Vancouver General Hospital (VGH) gastroenterology department's electronic medical records, which contain patient health records, colonoscopy reports, and pathology results. All patients in the study had a colonoscopy performed by one of three gastroenterologists at VGH.

To be eligible, patients must meet the following criteria: previous radiation treatment to abdomen, pelvis, spine, extended mantle field and/or whole-body irradiation; total amount of radiation is at least 10 Gy; first radiation is at least 10 years prior to first colonoscopy; age of the first colonoscopy is before 50 years of age; sufficient DNA tissue for molecular analysis; and at least 10% tumor cellularity.

Exclusion criteria include: history of inflammatory bowel disease (IBD), previous colonic polyps or colorectal cancer, a positive fecal immunochemical test result, a first-degree relative with CRC diagnosed before age 60 years, two first-degree relatives with CRC, the presence of an inherited CRC syndrome such as Lynch syndrome (hereditary non-polyposis colorectal cancer) or familial adenomatous polyposis, or colonoscopies with poor bowel preparations.

High-risk features were defined as: size of 10 mm or greater, three or more adenomas, tubulovillous or villous histology or adenomas with high-grade dysplasia.

A total of 18 patients with 22 colorectal adenomatous polyps were included in the study. For all the patients, the following information was recorded: the size, number, location (right colon is proximal to splenic flexure while left colon is distal to splenic flexure), histopatho-

logic features and findings of follow up colonoscopies. DNA was extracted from 2 x 10 micron scrolls of Formalin-fixed Paraffin-embedded (FFPE) tissue blocks using the Qiagen GeneRead DNA FFPE Kit. 75 ng of DNA was subjected to next generation sequencing using the 33 gene Find-It v3.4 cancer hotspot panel (Contextual Genomics, Vancouver, BC, Canada) and Ilumina MiSeq. The Find-it v3.4 cancer hotspot panel includes one hundred and forty-six hotspots and 23 exons in more than 30 cancer-related genes. The test detects single base substitutions (SNVs), deletions and insertion of up to 24bp.The genes tested include: (AKT1, ALK, AR, BRAF, CTNNB1, DDR2, EGFR, ERBB2, ESR1, FGFR1, FGFR2, GNA11, GNAQ, GNAS, HRAS, IDH1, IDH2, JAK1, KIT, KRAS, MAP2K1 (MEK1), MAP2K2 (MEK2), MET, NRAS, PDGFRA, PIK3CA, PTEN, POLE, PTCH1, RET, ROS1, SMO and TP53). The limit of detection was set at 5% variant allele frequency and 8500x mean coverage.

Results

NGS testing was performed on 18 polyps from 17 patients out of the 18 patients that were initially included, with one case failing due to suboptimal DNA quality. Only the mutations detected with the FindITtm Amplicon panel v3.4 that were present are listed. All other genes on the panel were negative for mutations. The result of the study is summarized in table 1.

Case#	Age	Sex	Location	Polyp #	Histology	Size (mm)	Mutation	Follow up status/months/ recurrence
1*	35	F	R	1	SSL	12		Alive/6 months/see case (2)
2*	35	F	L	1	CRC arising in a TA	11	TP53 (Intact MMR)	Alive/12 months/ TA and SSL (R)
3	35	M	R and L	3#	TA	8, 8 and 9		Alive/6 months/7 TAs (L)
4	49	F	R	1	TA	5		Alive/No follow up
5	35	F	L	1	TA	6		Alive/No follow up
6	38	F	R	1	TA	5		Alive/No follow up
7	35	M	R	1	TA	4		Alive/No follow up
8	44	F	R and L	2#	TA	5 & 3		Alive/No follow up
9	37	F	R	1	TA	6		Alive/No follow up
10	28	F	L	1	TA	3		Alive/No follow up
11	39	F	R	1	TA	6		Alive/No follow up
12	39	F	R	1	SSL	15	BRAF	Alive/35 months/no recurrence
13	27	F	R	1	TVA	10	KRAS	Alive/12, 22 and 34 months/ TA (L), TVA (L) and TA (L), respectively
14	41	M	L	1	TA	5		Alive/51 months/TA (L)
15	17	F	L	1	TA	30	KRAS	Alive/18 months/no recurrence
16	31	M	L	1	SSL	7	BRAF	Alive/59 months/SSL (L)
17	34	F	L	1	TA	10		Alive/No follow up
18	43	F	R	1	SSL	7		Alive/42 and 166 months/TA (L) and SSL (R), respectively

Table 1: *Summary of the clinicopathologic and NGS findings.*

*F: Female; L: Left Colon; M: Male; MMR: Mismatch Repair; R: Right Colon; SSL: Sessile Serrated Lesion; TA: Tubular Adenoma; TVA: Tubulovillous Adenoma; *: Same patient, two separate polyps, detected 6 months apart, respectively; #: Only one polyp tested.*

The median age of the patients that were tested using NGS is 35 years (range 17 - 49) at the time of colonoscopy. Thirteen patients were females (76.4%) and 4 patients were males (23.5%). The median age at first RT receipt was 10 years (1 - 16) and the median interval from radiation treatment was 28 years (10 - 43). The median total amount of RT exposure was 25 Gy (10 - 45). Four of the polyps had a histopathologic diagnosis of sessile serrated lesion (22.2%), while the remaining 14 polyps were either TA, TVA or TA with submucosal invasion (77.7%).

Of the 18 polyps tested, 5 polyps elucidated hotspot mutations at cancer-associated genes (27.7%), while the remaining 13 polyps were negative for the mutations tested (72.3%). Of the 5 mutations detected, 2 polyps showed substitution mutations of the KRAS gene at exon 2, codon G12C and G12S (c.34G>A and c.34G>T, respectively). Two polyps with histological diagnoses of SSL showed substitution mutations of the BRAF gene at exon 15, codon V600E (c.1799T>A). The last polyp had a histological diagnosis consistent with TA with submucosal invasion and elucidated a substitution mutation at the TP53 gene, exon 7, codon G245S (c.733G>A).

Histology, (n)	BRAF, n (%)	TP53, n (%)	KRAS, n (%)
SSL (n = 4)	2 (50%)		
TA with submucosal invasion (n = 1)		1 (100%)	
Low-risk adenomatous polyp (n = 8)			
Adenomatous polyps with HRF (n = 5)			2 (40%)

Table 2: *Correlation between histological and molecular findings.*
*HRF: High-Risk Features; *: Low-risk Tas.*

Discussion and Conclusion

Colorectal carcinoma is the most common gastrointestinal malignancy with well-understood molecular pathogenesis. The majority develop through the adenoma-carcinoma progression pathway with accumulative mutational burden. They starts as colorectal adenomatous polyps, the earliest precursor, and later acquire additional mutations leading to high-grade dysplasia followed by full-blown invasive carcinoma [10]. The earliest studied mutations include the APC and B-catenin pathway and result in early colorectal adenoma. On the other hand, KRAS, a frequently mutated gene in CRC, is acquired later in the pathway. It is most commonly seen in adenomas larger than 1 cm and in adenomas with high-grade features [11]. Importantly, TP53 mutations are usually the last acquired drivers resulting in invasive adenocarcinoma [12].

A more recently described alternative pathway to CRC tumorigenesis is the 'serrated pathway', characterized by mutations in KRAS and BRAF V600E, as well as epigenetic alterations of the CpG islands (CpG island methylator phenotype) [10]. Mismatch repair genes are another important initiators of both inherited and sporadic CRC. Other less frequent mutations implicated in CRC include PTEN, PIK3CA, SMAD4, SMAD2 and PPAR [13].

Several studies were conducted to determine the frequency of genetic mutations in colorectal adenomatous polyps. One of the recent studies is the Norwegian Colorectal Cancer Prevention (NORCCAP) Screening study [14], which analyzed 204 screening-detected polyps with the aim of assessing the frequency of oncogene mutations in BRAF, PIK3CA and KRAS. The study resulted in identifying KRAS mutations in (23.0%) of the lesions and is mostly associated with larger size and adenomas with high-grade features. Additionally, BRAF mutations were identified in (11.3%) showing high association with serrated morphology. Only 2 cases (1%) were found to harbor mutations in PIK3CA.

Another study from Spain by Juarez., *et al*. [11] found similar numbers when they assessed the frequency of KRAS and BRAF mutations in colonic polyps and its association with metachronous neoplasia. They included 995 polyps, with all the polyps exhibiting high-grade

features (defined as either size >= 10 mm, high-grade dysplasia or villous component). BRAF mutations were identified in (14.9%) of the polyps, most of which are serrated. KRAS mutations were elucidated in (22.9%) of the polyps and where found in both serrated and non-serrated polyps. They concluded that KRAS mutations are associated with the development of metachronous advanced polyps and advanced adenomas. It is of note that the study only included polyps with high-grade features.

Another study by Yan., *et al*. [12] assessed the frequency of KRAS and TP53 in colorectal neoplasms. It included four groups: patients with colorectal adenomas, patients with single primary CRC, patients with multiple primary CRC and a control group with healthy individuals. The study showed an increase in the frequency of TP53 and KRAS mutations in patients with established invasive carcinoma than in the group with colorectal adenoma.

As previously demonstrated by T. O. Henderson., *et al*. [7] childhood radiation survivors are at a higher risk adenomas and CRC. The is significant data limitation in the literature of studies assessing the mechanism of tumorigenesis in these patient population. One of the potential mechanism proposed by Kim SB., *et al*. [15] in 2016, is radiation-induced senescence associated inflammatory response (SIRS). Using a mouse model, they demonstrated that proton irradiation increased the expression of senescence-associated inflammatory response (SIR) genes, effectively creating an inflammatory milieu, leading to DNA damage and TP3 mutations. Interestingly, they raise the possibility of pre-treating radiation patients with NSAIDs, which theoretically could lead to the reduction of SIRS. Further studies will have to be conducted to assess response to those agents.

Our study is one of the first to use NGS to analyze adenomatous polyps in childhood cancer survivors treated with radiation therapy. We found that 2 polyps (11.1%) harbored activating mutations in the BRAF V600E gene and 2 other polyps (11.1%) elucidated activating mutations in the KRAS gene at exon 2. These numbers are slightly less than those seen in previous international studies including the NORCCAP study [14]. This is likely attributed to the small sample number in our cohort. All the polyps that elucidated KRAS mutations showed high-grade features, with one showing tubulovillous morphology and the other showing a tubular adenoma measuring 30 mm. This represents 40% of the polyps with high-grade features in our sample. In addition, all the polyps (100%) that harbored mutations in BRAF V600E were sessile serrated lesion (SSL), representing 50% of all the serrated polyps in our sample. Both findings are concordant with previous literature findings, which established that KRAS mutated polyps exhibited higher grade features than nonmutated polyps. In addition, BRAF mutations in our study were only detected in serrated polyps, also in agreement with the previous literature.

Interestingly, while TP53 is a frequent mutator in radiation-associated sarcomas and solid carcinomas [16], our study only detected one TP53 mutated polyp (5.5%) and is the only polyp with submucosal invasion in our cohort. These findings confirm that KRAS and TP53 are late driving forces in progression to carcinoma in both the general population and in radiation-associated polyps.

In our study, we found close association between the morphology of radiation-associated colorectal adenomas and their mutational profile. These association are similar to adenomas in the average-risk population, and thus, likely to follow the conventional 'adenoma-carcinoma' pathway or the alternative 'serrated' sequence, albeit at an accelerated rate. Also, no novel driving mutations in other cancer-associated genes were detected in the 18 polyps we analyzed using the NGS panel. Testing for Wnt signaling pathway (APC/B-catenin), which plays a role in adenoma initiation and progression, was negative in all our cases.

One of the limitations of our study is that it did not include the mismatch-repair (MMR) genes and will have to be included in future studies. Our findings are also limited by the small sample size and must be confirmed by prospective studies of larger cohorts with validation arm and by using more comprehensive molecular testing.

This retrospective study is one of the first to molecularly characterize colorectal adenomatous polyps in childhood cancer survivors treated with radiation therapy using Next Generation Sequencing. Our molecular analysis demonstrate that radiation-induced polyps harbor similar genetic changes to dysplastic adenomatous polyps in the average-risk population. We postulate that radiation-associated

polyps follow the conventional 'adenoma-carcinoma' pathway or the alternative 'serrated' sequence, making them amenable to detection and management by early colonoscopy. Furthermore, our NGS panel failed to detect novel driving mutations in cancer-associated genes. More comprehensive molecular analysis of these polyps is required to understand their molecular composition and to aid in identifying actionable genetic alterations.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

The study was approved by the appropriate institutional and/or national research ethics committee and was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. For this type of study formal consent is not required.

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