Hypermethylation of RAD9A intron 2 in childhood cancer patients and tumor cell lines

CURRENT STATUS: POSTED

Danuta Galetzka
University Medical Centre Johannes Gutenberg University Mainz

Danuta.Galetzka@unimedizin-mainz.de Corresponding Author
ORCiD: https://orcid.org/0000-0003-1825-9136

Julia Böck
Institute of Humangenetics Julius-Maximilians-University Würzburg

Marcus Dittrich
Institute of Human Genetics Julius Maximilians University Würzburg

Iris Schmitt
Department of Radiation Oncology and Radiation therapy university Medical Centre Mainz

Olesja Sinizyn
Department of radiation oncology and Radiation Therapy University Medical Centre Mainz

Marco Ludwig
DRK Medical Center Alzey

Heidi Rossmann
Institute of Clinical Chemistry and Laboratory Medicine, University Medical Centre

Claudia Spix
German Childhood Cancer Registry Institute of Medical Biostatistics Epidemiology and Informatics university Medical Centre Mainz

Peter Scholz-Kreisel
Institute of Medical Biostatistics, Epidemiology and Informatics University Medical Centre Mainz

Johanna Mirsch
Radiation Biology and DNA Repair, Darmstadt University of Technology

Matthias Linke
Institute of Human Genetics University Medical Centre Mainz
Dirk Prawitt  
Center for Pediatrics and Adolescent Medicine University Medical Center Mainz

Manuela Marron  
Leibniz Institute for Prevention Research and Epidemiology-BIPS, Bremen

Alicia Poplawski  
Institute of Medical Biostatistics, epidemiology and Informatics, University Medical Centre Mainz

Thomas Haaf  
Institute of Human Genetics, julius Maximilians University Würzburg

Heinz Schmidberger  
Department of radiation Oncology and Radiation therapy university Medical Centre Mainz

DOI: 10.21203/rs.2.11904/v1

SUBJECT AREAS
Oncology

KEYWORDS
RAD9A, childhood cancer risk, hypermethylation, normal body cells, somatic mosaicism
Abstract
Background Most childhood cancers occur sporadically and cannot be explained by an inherited mutation or unhealthy lifestyle. The prenatal origins hypothesis postulates a role for (epi)genetic mutations which occur stochastically in rapidly dividing cells. This study aims to investigate the impact of adverse methylation in tumor relevant genes in former childhood cancer patients which may be associated with an enhanced risk to develop primary secondary cancers Methods We performed an epimutation screen of several candidate genes (APC, CDKN2A, EFNA5, RAD9A, and TP53), in skin fibroblasts of 20 patients with a primary cancer in childhood and subsequent second primary cancer (2N), 20 matched patients with only one primary cancer in childhood (1N), 20 cancer free controls (0N) and unrelated leukemia cancer samples, using bisulfite pyrosequencing and deep bisulfite sequencing. radiation, colony formation assays, cell proliferation, PCR and molecular karyotype SNP-array, experiments were performed to further characterize RAD9A hypermethylation in fibroblasts and FaDu sub clones. Data were analyzed using the Kruskal-Wallis rank sum test and Benjamini-Hochberg procedure, the linear mixed-effects model was fit using REML and R-scripts. Results Four 1N patients and one 2N patient displayed elevated (≥10%) mean methylation levels of RAD9A intron 2. Deep bisulfite sequencing of RAD9A in these patients revealed ≥2% allele methylation errors (defined as alleles with ≥60% methylated CpGs). We found RAD9A hypermethylation in bone marrow of patients with pre-ALL (pre-acute lymphoblastic leukaemia), AML (acute myloid leukaemia), and plasmablastic lymphoma (PBL), and in EBV-(Epstein Barr virus) transformed lymphoblastoid cells. RAD9A methylation in fibroblasts or tumor cells (FaDu) was not affected by in vitro aging, or DNA damage induced by radiation or the chemotherapeutical daunorubicin. Molecular karyotyping of FaDu sub clones revealed a homozygous inactivation of CHD2, SPATA8 and SMARCA1, in clones with hypermethylation in RAD9A. Conclusion We propose that constitutive RAD9A epimutations may have arisen early in development, predisposing the compromised cells to tumorigenesis and increased childhood cancer risk. Analyses of tumor cell clones with high methylation levels of RAD9A suggest a connection between methylation levels of the RAD9A intron 2 locus and a homozygous inactivation of important genes.
Background
Tumorigenesis is a multistep process, involving an accumulation of genetic and epigenetic changes in multiple genes resulting in both, the, inactivation of tumor suppressor (TS) genes and/or activation of oncogenes [1, 2]. Tumor epigenomes are characterized by a global loss of DNA methylation, leading to reactivation of retrotransposons and genome instability, as well as regional hypermethylation and silencing of TS genes, compromising DNA repair and cell cycle control [3, 4]. In sporadic cancer (epi)genetic changes, which may arise by stochastic DNA replication errors or adverse environmental exposures, are usually restricted to the tumor and its precursor cells. In contrast, most hereditary forms of cancer are caused by germline mutations in tumor suppressor genes, which predispose patients to tumor development, which itself is triggered by inactivation of the second TS allele.

Accumulating evidence suggests that, similar to germline mutations, constitutive epimutations involving soma-wide hypermethylation of tumor suppressor genes in normal body cells, can cause phenocopies of cancer syndromes such as hereditary non-polyposis colon cancer (HNPCC) [5, 6] as well as breast- and ovarian cancer [7, 8]. Since constitutive epimutations usually occur in a mosaic state with variable proportions of affected cells in different tissues, they are most likely not transmitted through the germline but may arise during early development. For some cancer-predisposing genes, i.e. MLH1 [9], MSH2 [10], and DAPK1 [11], the probability for de novo epimutations depends on cis-regulatory genetic sequence variants.

Compared to the aging population, cancer is rare among children and young adults, representing <1% of all cancers. Children are usually not exposed to an unhealthy lifestyle or an adverse environment, and only 5–10% of children with cancers carry germline mutations increasing their cancer risk [12]. Therefore, most childhood cancers should occur sporadically. One explanation for sporadic childhood cancers is somatic mosaicism. A high proportion of human preimplantation embryos are chromosomal mosaics of normal and aneuploid cells [13]. One embryonal cell carrying a de novo chromosome or genetic mutation can be propagated into different tissues and organs during somatic development, laying dormant for many years. Approximately 30% of all human tumors are endowed with RAS mutations, consistent with an essential role of the RAS signaling pathway for
tumorigenesis. Several RASopathies including neurofibromatosis type 1 (NF1), Noonan syndrome, Costello syndrome (CS) and cardiofaciocutaneous syndrome (CFCS) are associated with increased cancer risks [14, 15]. Although most congenital RASopathies are caused by germline RAS mutations, NF1 and other RASopathies can also be present in a mosaic state, due to postzygotic or somatic mutations.

The prenatal origins hypothesis postulates that childhood cancers arise from postnatally persisting embryonal or more differentiated prenatal cell with predisposing mutations [16, 17]. Several studies have demonstrated prenatal oncogenic events underlying acute leukemia in childhood [18–21]. During each cell division not only the DNA sequence but also its epigenetic modifications are copied to the daughter cells. Considering that the error rate during this copying process is at least one magnitude higher for epigenetic information than for genetic information [22], constitutive epimutations may occur much more frequently than prenatal DNA sequence mutations. We have previously described monozygotic twin sisters discordant for childhood cancer and a constitutive epimutation in the BRCA1 TS gene [23].

To analyze the role of constitutive epimutations in childhood cancer, we performed an epimutation screen for several cancer-relevant genes in a unique cohort, consisting of fibroblasts derived from individuals who survived childhood cancer and subsequently developed a second primary cancer (2N) and matched individuals with childhood cancer but without a second cancer (1N). Moreover, fibroblasts of 20 matched, cancer-free individuals served as controls (0N).

**Materials And Methods**

**Patient samples and cell lines**

This study was approved by the Ethics Committee of the Medical Association of Rhineland-Palatinate (no. 837.440.03 [4102]; no. 837.262.12(8363-F); and 837.103.04 (4261)]. Written informed consent to use primary fibroblasts for research purposes was obtained after genetic counselling for all participating patients. With the help of the German Childhood Cancer Registry, 20 individuals who survived a childhood malignancy and then developed a second primary cancer (2N) and 20 carefully matched (first tumor, manifestation age, sex) individuals who survived a childhood cancer without
developing a second malignancy (1N) were recruited for the KiKme study (Cancer in Childhood and Molecular Epidemiology) [69]. Twenty matched patients (sex and age) without cancer from the Department of Accident Surgery and Orthopedics served as controls (0N). All patients were followed up from primary cancer diagnosis to recruitment for this study several years after treatment. The primary malignancies included, 11 patients suffered from acute lymphatic or myeloid leukemia, 5 patients from Hodgkin- or Burkitt Lymphoma, and 4 patients from solid tumors. The second cancers in the 2N group were myelodysplastic syndrome, lymphoma, thyroid cancer and solid tumors. Affymetrix array analysis [40] of 1N and 2N patients did not reveal detectable copy number variations in known tumor suppressor genes (BRCA1, BRCA2, RAD9A, TP53, NF1) and oncogenes (PTPN11, ETV6-RUNX1, TCF3-PBX1). No pathological germline TP53, RAD9A and BRCA1, 2 mutations were identified using Sanger sequencing (TP53 Mutation Database (http://p53.iarc.fr/); https://www.ncbi.nlm.nih.gov/clinvar/ and https://www.lovd.nl/) (all patients). RB1 gene analysis in patient 1N20 did not reveal pathological mutations. By bisulfite pyrosequencing, none of the matched (1N or 2N) childhood cancer patients showed BRCA1 hypermethylation, consistent with an epimutation [23]. Cell lines 0N24 and 2N24 are a pair of discordant monozygotic twins. One twin suffered from childhood leukemia and later on from a thyroid carcinoma whereas her sister was completely healthy until adulthood. Control fibroblast strains for cell proliferation and senescence analyses were established from excess skin materials from surgical interventions in non-cancer patients [70].

Epstein Barr virus (EBV) transformation of resting B cells (in peripheral blood lymphocytes) to proliferating lymphoblastoid cells occurs in early stages of infection and is widely used to obtain immortalized lymphoblast cell lines [49]. Lymphoblastoid cells were harvested in an early passage after stable infection. CVS-Samples (excess material) were obtained during the course of routine diagnostics. Bone marrow samples (excess material from routine chromosome diagnostics) were obtained from patients with pre-B-ALL (46,XY,t(9;22)(q34;q11.2)[2]/46,XY[25], AML (46,XY,der(7)(q-)
.ish del(16)(q22)[12]/ 46,XY[10], and plasmablastic lymphoma (PBL) with complex aberrations in 60% of cells, consisting of a hypodiploid (28%) clone and a hyperdiploid line (32%), which arose through
duplication of the hypodiploid line. The FaDu tumor cell line, carrying mutations in CDKN2A (c.151–1G>T), TP53 (c.376–1 G>A; c.743G>T) and SMAD4 (c.1_1659del1659) was purchased from ATCC.

**Cell culture and irradiation**

Primary fibroblasts from skin biopsies and the FaDu tumor cell line were cultured in Minimal Essential Medium with Earle’s salts (Invitrogen, Karlsruhe, Germany), supplemented with 15% fetal bovine serum (FBS) Fa. Merk, Darmstadt, 1% vitamins and 1% antibiotics (Pen/Strep) in a 90% humidified incubator with 5% CO₂ at 37°C. All experiments using the primary fibroblasts were performed with growth-arrested cells in the G0/G1 stage. Confluency was achieved by contact inhibition and subsequent cultivation for 2 weeks and confirmed by FACS (flow cytometric cell cycle) analysis. For comparisons of 2N, 1N, and 0N patients, fibroblasts with a similar passage number 5 (±2) were used.

Cells were exposed to X-rays with a D3150 X-Ray Therapy System (Gulmay Ltd, Surrey, UK) at 140 kV and a dose rate of 3.62 Gy/min at room temperature. Sham irradiated control cells were kept at the same conditions in the radiation device control room. The FaDu tumor cell line was irradiated at 80% of confluency. Cells were exposed to single doses ranging from 2–8 Gy and harvested at 15 min, 2 h, and 24 h after irradiation. For fractionalized irradiation, fibroblasts were irradiated 4x, 8x, or 10x within a period of 20 days with doses of 2 Gy and 4 Gy, respectively, within a period of 20 days. Cells were given one day of recovery time between exposures and the medium was changed twice a week. Cells were harvested one day after the final irradiation.

Cell lines MCF7, ZR–75–1, EFO27 und T47D were cultivated in RPMI1640 (Gibco) supplemented with 10% FBS, 2.5% HEPES buffer (Sigma) and 1% antibiotics (Pen/Strep) (Life Technologies). The A549 cell line was cultured in DMEM modified with 10% FCS.

FaDu derived single cell clones were generated by dilution of the primary cell line and propagated in Minimal Essential Medium with Earle’s salts (Invitrogen,), supplemented with 15% FBS, 1% vitamins and 1% antibiotics (Pen/Strep) using 96 plates (Cellstar). After 48h wells with only one cell were selected via microscopic examination. Subsequent propagation was performed in conditioned medium (one day old medium of primary culture at 50% confluency, sterile filtered using 0,2µm filter) and cells were transferred in in to a 24 well plate, later a 6 well and finally 10 cm petri dishes when they
reached 80% confluency.

**Daunorubicin treatment**

Fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 1% vitamins and 1% antibiotics (Pen/Ptrep) in a 90% humidified incubator with 5% CO\(_2\) at 37°C. The cells were cultured in T25 flasks to 80-90% confluence, than a non-lethal dose [48] of 3µM daunorubicin (Pfizer Pharma PFE GmbH) was added. After 2 hours the medium was replaced and at time points of 2 h, 4 h and 24 h post treatment the cells were harvested by trypsinization. Quantification of γH2AX was performed as previously described [71].

**Replicative senescence culture**

Fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 1% vitamins and 1% antibiotics (Pen/Strep) in a 90% humidified incubator with 5% CO\(_2\) at 37°C. Cells were cultured and passaged, every two weeks at 80–90% confluence. Cells were harvested at passages p7, p17 and p25, starting with the p5 after biopsy. At p25 all cells had reached replicative senescence (Fig. 2).

**Growth kinetics of FaDu and sub clones**

Tumor cell lines were cultured as described above. Cells were collected and seeded at 5*10^4 density in a T75 cell culture flask in triplicates. The cells were harvested by trypsinization and total cell numbers were determined by counting using Moxi\(^2\) automatic cell counter Fa. Orflo, at every time point. The cellular proliferation rate was calculated as cumulative population doublings (CPD). The statistical analysis was done using the linear mixed-effects model fit by REML setting the biological replicate as random variable.

**Colony formation assay**

Clonogenic survival was determined in colony formation assays adapted after Menegakis et al [72]. At passage 8, cells were seeded (1x10^5) in 10 cm diameter petri dish in triplicates. After 5 days and one change of medium, the cells were irradiated with 2 Gy, 4 Gy, 6 Gy and 8 Gy. Sham irradiated cells were kept at the same conditions in the radiation device control room (0 Gy). After 24 h cell
suspensions were obtained for each dose and different seeding densities were plated in triplicates. Remaining cells were pelleted, washed with PBS and stored at -80°C for further experiments. 14 days after irradiation colonies were fixed and stained with crystal violet. Colonies defined as >50 cells were counted and surviving fractions were expressed in terms of plating efficiency. Survival data after radiation dose were fitted to linear quadratic regression models employing the maximum likelihood approach (r package CFAssay). Differences between curves were evaluated using the F-test. Adjustment of p-values was done using the method of Benjamini and Hochberg. Image of a FaDu cell line (0Gy) was made with the ZEISS Axiovert microscope using the Clone software at 10x magnification.

**SNP CGH (molecular karyotype) analysis**

High-resolution screening for microdeletions and duplications was performed with the Affymetrix GeneChip Genome Wide Human SNP Cyto HD, following the protocol developed by the manufacturer (Affymetrix, Santa Clara, CA, USA). Data calculation was performed with Chromosome Analysis Suite 3.1.0.15 and NetAffx Build 33.1 (hg19)

**Flow cytometry**

Cells were washed with PBS (37°C; 1ml/25cm²) and trypsinzed (Trypsin/EDTA (37°C; 1 ml/ 25 cm²), 5 min 37°C). The reaction was stopped by the addition of a 2-fold volume of culture medium at 37°C and cells were collected by centrifugation at 300 x g for 6 minutes. 70% ethanol (–20°C) was added dropwise to the cell pellet under permanent turbulence (~ 4 ml/10⁵ cells) and the suspension was stored for ≥ 30 min at 4°C or overnight. Later, the cell suspension was centrifuged at 300 x g for 6 minutes, and the supernatant was discarded. Cells were re-suspended in PBS (without magnesium and calcium -/-) with RNase (20 µg/ 10⁴ cells) and incubated 30 min at 37°C. After an additional centrifuge step of 300 x g for 6 minutes, cells were re-suspended in HOECHST 33258-staining solution (0.2 µg/ml in 1x PBS/-) (~ 4 ml/ 10⁵ cells) and again incubated for ≥ 30 min at 4°C. Analysis was performed using FaCS Canto II Fa. BD Biosciences.

**PCR and sequencing**
Genomic DNA was isolated using the NucleoSpin tissue kit (Macherey-Nagel, Germany). The PCR reactions were performed using the FastStart Taq DNA Polymerase, dNTPack, kit # 04738403001 (Fa. Roche). PCR was performed in three stages with one cycle of 5 min at 94°C, forty cycles (15 sec, 94°C, 30 sec TM, 1 min 68°C) and one cycle of 2 min at 72°C, using 30 ng DNA per reaction. Primers were generated using, NCBI’s (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) primer designing tool. The following primer pairs were used; forward primer (AGGCAGTCAGTCGAAAGTG) and reverse (TTGGAACCTGCTGATTSG) for CHD2 (NM_001271) TM 62°C; forward primer (ACAGCTGTTTCTCAGGAAGG) and reverse (TAGGCTGCGAGGATGCTT) for SPATA8 (NR_158221) TM 62°C and forward Primer (GCAAGAAGATGAGAAGC) and reverse (GGCAGGTAAGCTCAGGGT) for SMARCA1 (NM_003069) TM 64°C. Sequence reactions were performed by Fa. Genterprise Germany, using PCR products digested with Exo/SaP (Fa. New England Biolabs).

**Bisulfite Pyrosequencing**

Genomic DNA was isolated with the NucleoSpin tissue kit (Macherey-Nagel, Germany). Bisulfite conversion of 0.2 µg DNA was performed with the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR and sequencing primers for the genes above-mentioned were designed with PyroMark Assay Design 2.0 software (Qiagen) (Supplementary Table 1). The 25 µl PCR reactions consisted of 2.5 µl 10x PCR buffer, 20 mM MgCl₂, 0.5 µl dNTP mix (10 mM), 1 µl of each forward and reverse primer (10 µM), 0.2 µl FastStart Taq DNA Polymerase (5 U/µl) (Roche Diagnostics, Mannheim, Germany), 18.8 µl PCR-grade water and 1 µl (~100 ng) bisulfite-converted template DNA. PCR amplifications were performed with an initial denaturation step at 95°C for 5 min, 35 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 45 sec and a final extension step at 72°C for 5 min. Bisulfite pyrosequencing was performed on a PyroMark Q96 MD Pyrosequencing System using the PyroMark Gold Q96 CDT Reagent Kit (Qiagen) and 0.5 µl of sequencing primers (10 mM). Data analysis was performed with the Pyro Q-CpG software (Qiagen). Data were analyzed using Kruskal-Wallis rank sum test. We considered the differences only statistically significant after adjustment of p-value by method of Benjamini and Hochberg.

**Deep bisulfite sequencing (DBS)**
Next-generation sequencing (NGS) libraries for DBS were generated as described previously [8]. PCR amplification of \textit{APC}, \textit{CDKN2A}, \textit{TP53}, and \textit{RAD9A} was performed using primers containing a target-specific part and partial adapter overhangs (Supplementary Table 2). Following purification of the amplicons with magnetic beads (0.9:1), DNA concentration was determined with the dsDNA BR Assay System (Life Technologies, Carlsbad, USA). Samples were diluted to 0.2 ng/µl in a total volume of 15 µl each. After pooling, barcoding was performed by PCR with NEBNext Multiplex Oligos for Illumina, Dual Index Primer Set 1 (New England BioLabs, Frankfurt a. M., Germany), followed by another bead cleaning step (0.9:1). DNA concentration and fragment length were determined with the High Sensitivity DNA kit on a 2100 Bioanalyzer (Agilent Technologies). All barcoded pools were diluted to 2 nM and pooled to the final library. Denaturation and preparation of the library and PhiX control were done according to the manufacturer’s protocol (Illumina, San Diego, USA). Paired-end sequencing was performed on an Illumina MiSeq using the MiSeq Reagent Kit v2 (2 x 150 cycles) cartridge.

\textbf{DBS Analysis}

The sequences in FASTQ format were processed using the Amplikyzer2 [73] pipeline, which provides a detailed nucleotide-level analysis including the calculation of CpG methylation rates. All sequences were aligned to the genomic sequence of each amplicon using default settings. For the subsequent extraction of read and CpG-wise methylation status only reads with an overall bisulfite conversion rate of over 95% have been considered. Further downstream processing of Amplikyzer2 output files and subsequent analyses of methylation rates was performed using R-Scripts. Statistical analyses were performed with the statistical software package R (Version 3.2.2) [74].

\textbf{Results}

\textbf{Selection of genes for an epimutation screen}

We aimed to analyze gene methylation of the most likely candidate genes which are involved in cancer. No pathological germline \textit{TP53}, \textit{BRCA1}, \textit{BRCA2} and \textit{RAD9A} mutations were detected using Sanger sequencing in our patients. According to Magnusson et al., [24] there is a connection between familial mutations in BRCA1/2 and CDKN2A genes and childhood cancer. Although we do not have any information about the mutational status of family members, the disease spectrum seen in our
patients matches the collective screened in the study conducted by Magnusson and colleagues. Germline mutations in the TS gene BRCA1 account for 25–30% of hereditary breast- and ovarian cancer. It plays a major role in homologous recombination and other DNA repair mechanisms [25]. BRCA1 haploinsufficiency may induce genomic instability and premature senescence in breast epithelial cells [26]. The CDKN2A TS gene is deleted, mutated or epigenetically silenced in a wide variety of cancers [27, 28]. It inhibits the catalytic activity of the CDK-cyclin D complex, a regulator of the G1 checkpoint of the cell cycle [29]. Mutations in the APC TS gene cause familial adenomatous polyposis and colon cancer [30]. Inactivation of APC promotes tumorigenesis through loss of cell adhesion [31] and chromosome instability [32]. Aberrant signaling of the Eph family of receptor tyrosine kinases and their ephrin ligands have also been implicated in human cancers. Aberrant methylation of EFNA5 have been found in acute lymphocytic leukemia [33] and non-Hodgkin lymphoma [34]. Germline mutations in the TP53 TS gene cause Li-Fraumeni syndrome which is characterized by familiar occurrence of childhood soft tissue sarcomas and a wide spectrum of early-onset cancers. Somatic mutations in TP53 are among the most frequent genetic alterations in cancer [35]. Mutations in TP53 exon 6 can lead to truncated protein with a putative oncogenic gain of function in cancer cells [36].

The RAD9A gene is involved in different DNA repair pathways, including base excision repair, homologous recombination and mismatch repair, alongside multiple cell cycle phase checkpoints and apoptosis [37]. At least some of these functions are mediated by the RAD9A-HUS1-RAD1 complex. Overexpression due to gene amplification or intron 2 hypermethylation is found in a variety of cancers, consistent with an oncogenic function [38, 39]. On the other hand, reduced expression levels in normal body cells of childhood cancer patients with a second primary tumor also suggest a function as genomic caretaker [40].

**Epimutation screen of fibroblasts derived from childhood cancer patients**

Using bisulfite pyrosequencing, we have determined mean methylation of the promoter regions in APC, CDKN2A, and EFNA5, a cis-regulatory region in RAD9A intron 2, and a mutation hotspot in TP53.
exon 6 in 20 primary fibroblast cell lines of cancer free controls and 20 1N and 20 2N matched patients (Figure 1). There were significant variation in methylation among the groups. APC (Figure 1A) proved to be hypermethylated in 1N in comparison to the 0N control (p value = 0.03). CDKN2A (Figure 1B), which is often mutated in a variety of cancers, exhibited hypermethylation in the 1N group (p value = 0.006) while it was hypomethylated in 2N group in comparison to 0N group (p value = 0.008). The 2N group showed a hypomethylation in TP53 (Figure 1E) in comparison to the control group 0N (adj. p value = 0.01). No significant differences between the groups, were detected for EFNA5 (Figure 1C) and RAD9A genes (Figure 1D). In a previous work we showed that outliers could be considered as likely candidates for an abnormal methylation pattern, indicative for a mosaic epimutation [23]. We identified one patient (1N08) who exhibited a conspicuous hypermethylation (9%) of the APC promoter, while patient 1N15 showed a hypermethylation of CDKN2A (7%), and patient 2N12 displayed hypomethylation in TP53 (95%). Five patients (1N04, 1N07, 1N14, 1N20, and 2N21) showed increased RAD9A intron 2 mean methylation, ranging from 10% to 31%. The results for the analyzed outliers are compiled in Table 1.

Average methylation of millions of DNA molecules in a genomic DNA sample is a surrogate marker which can sometimes be difficult to interpret. Methylation changes could be due to either single CpG methylation errors at different positions in a large number of alleles or to a few allele methylation errors, where all or most CpGs in individual DNA molecules are aberrantly methylated. Because it is usually the density of CpG methylation in a cis-regulatory region rather than individual CpGs that turns a gene “on” or “off” [41, 42], allele methylation errors must be considered as putative functionally relevant epimutations. Deep bisulfite sequencing (DBS) can determine the methylation profiles of many thousands of individual DNA alleles for multiple genes and samples in a single experiment and, thus, directly measure epimutation rates (EMRs). In the present study we performed DBS on the patients with suspected Epimutations (Table 1). Alleles with >50% aberrantly (de)methylated CpGs in DBS are considered as functionally relevant epimutations. Using this classification patient 1N15 displayed 0.2% CDKN2A and 2N12 0.3% TP53 epimutations. We did not detect any APC epimutations in 1N08. In contrast, the 5 patients with suspected RAD9A epimutations
displayed 2.0% to 24.5% hypermethylated (>60%) alleles (Table 1). Consistent with an epimutation screen in breast cancer susceptibility genes [8], we considered EMRs >1% as elevated and EMRs >2.5% as likely pathogenic constitutive epimutations. Using this classification, childhood cancer patient 1N04 showed an elevated RAD9A EMR and 4 patients (1N07, 1N14, 1N20, and 2N21) RAD9A epimutations. Overall, 10% (4 of 40) childhood cancer patients (1N and 2N) had RAD9A epimutations in their normal cells.

**Effects of aging, radiation and chemotherapeutics on RAD9A methylation**

As most of our patients (except 1N20) did receive chemo- and radiotherapy during treatment and the donation of the fibroblast was done in adulthood, several years after the first malignancy, we designed experiments which may indicate treatment related changes in the methylation of our studied genes.

**DNA methylation during in vitro aging**

Cellular aging in vitro has been frequently used to study cancer pathologies that are caused by accelerated/premature molecular aging [43]. A recent methylation array analysis revealed >500 genes with significant methylation changes during extended fibroblast culture [44]. To study age or tissue culture effects on methylation, fibroblast strains of controls (0N01, 0N02, 0N03, and 0N24) and childhood cancer patient 2N024 were propagated in culture from passage 7 until senescence. Strain 0N03 showed the lowest (n = 20) and 0N01 (n = 41) the highest number of population doublings (Fig. 2A). Methylation of *APC*, *BRCA1*, *CDKN2A*, and *RAD9A* was measured at passages 7, 17 and 25 by bisulfite pyrosequencing. None of the analyzed genes showed a correlation between mean methylation (measured by bisulfite pyrosequencing) and in vitro aging (Fig. 2B-F).

**Effects of radiation**

We have shown previously that DNA methylation remains rather stable in primary fibroblasts within the first cell cycle after irradiation [45]. In contrast, significant methylation changes in >250 genes and the MAP kinase signaling pathway were associated with delayed radiation effects in irradiated fibroblast single cell clones [44]. To study radiation effects on the methylation of *RAD9A* intron 2 site,
the control cell line 0N18 was analyzed at 15 min, 2 h and 24 h after irradiation with 0 Gy, 2 Gy, 5 Gy, and 8 Gy, at each time point. RAD9A methylation values remained virtually unchanged between 7% and 9% (Table 2). Furthermore three fibroblast strains (0N12, 1N08, and 2N12) were irradiated in fractions of 8x 2 Gy, 4x 4 Gy, 10x 2 Gy, 8x 4 Gy, and/or 10x 4 Gy within a 20 day period. Again RAD9A methylation remained rather constant at 5% in 1N08 and 2N12, and at 8-9% in 0N12 (Table 2). In addition, exponentially growing FaDu tumor cells were analyzed at 2 h, 4 h, and 24 h after irradiation with a single dose of 0 Gy, 2 Gy, 5 Gy, and 8 Gy, respectively. RAD9A methylation varied within a narrow range between 54% and 57% (Table 2) and there was no difference between irradiated and non-irradiated cells. The proportion of G2-phase cells (measured by flow cytometry for FaDu cells) increased with radiation dose and time after irradiation, ranging from 35% in non-irradiated cells to 65% at 24 h after 8 Gy indicating a functional G2/M checkpoint. Data are shown in supplementary Figure 1.

**Effects of daunorubicin treatment**

Although tumor therapy varied between patients, daunorubicin and doxorubicin were frequently used in the treatment regimens. As both drugs have similar properties [46] and the cellular uptake of daunorubicin is superior to that of doxorubicin [47], we analyzed the influence of daunorubicin, on the RAD9A methylation. Treatment of normal fibroblasts with 3µM danorubicin, as stated in the study of Przybylska and colleagues [48], yields 60% of surviving cells. Analysis of γH2AX as a marker for DNA double-strand breaks confirmed the incorporation and toxicity of daunorubicin in the cells. Examination of the methylation signature at 0 h, 1 h, 4 h, 12 h and 24 h post treatment showed no significant changes in methylation in two independent fibroblast cell lines. 2N24 cell line derived from a second cancer patient exhibited mean methylation values of 4–6% and 0N24 control cell line of 9–12%. Data are shown in supplementary Figure 1.

**RAD9A hypermethylation during EBV transformation and tumor development**

According to Cheng et al., [38] RAD9A becomes an oncogene via hypermethylation in intron 2. This means if a cell became transformed toward unlimited growth and shows distinct tumor
characteristics, changes in methylation values in intron 2 may be detectible. Oncogenic transformation of B cells results in unlimited growth and has been associated with particular forms of cancer, such as Burkitt’s lymphoma, Hodgkin’s lymphoma, mononucleosis, nasopharyngeal carcinoma, and gastric cancer. EBV particles were found in cultures derived from Burkitt’s lymphoma and EBV was the first human tumor virus identified. Thus EBV infection is now widely used to generate immortal lymphoblastoid cell lines [49]. Global changes in DNA methylation may contribute to the pathogenesis of EBV [50]. We therefore tested EBV transformed lymphoblasts for changes in methylation of RAD9A in intron 2. The mean methylation varied in six different EBV transformed cell lines from 6% to 41% (data shown in supplementary Figure 1. 3). The bisulfite next generation sequencing (NGS) analysis of the cell line with the highest methylation value using bisulfite NGS exhibited 41% RAD9A methylation and 9% fully methylated alleles. In contrast, the methylation patterns of APC, BRCA1, CDKN2A, and TP53 in this cell line remained virtually unchanged after transformation (shown in supplementary Table 3).

To corroborate the results of leukemic transformation, we additionally analyzed methylation in the bone marrow of three leukemia patients. Patient 1 with pre-ALL and a Philadelphia chromosome in <10% of analyzed (bone marrow) cells displayed a mean RAD9A methylation of 18%. Patient 2 with AML and 50% bone marrow cells with abnormal karyotype displayed 29% RAD9A methylation. Patient 3 with PBL and 60% cells showing complex aberrations consisting of a hypodiploid (28%) clone and a hyperdiploid line (32%), had a RAD9A methylation of 41% (data shown in supplementary Figure 1. 3).

**Methylation of RAD9A in CVS (Chronic villus sampling) samples**

To elucidate if changes in chromosomal numbers, as often seen in leukemic cells, may induce aberrant methylation in the RAD9A locus, we analyzed nine CVS derived cell lines with normal karyotypes and six cell lines with chromosomal imbalances detected by prenatal routine diagnostics. CVS is a diagnostic test for identifying chromosome abnormalities and other inherited disorders. It involves removing some chorionic villi from the placenta at the point where they attach to the uterine wall. The cytogenetic karyotype resembles that of the fetus. Miscarriages are sometimes the result of
intrauterine infections or chromosomal imbalances. Conspicuous cell lines included trisomy (47,XY+22; 47,XX+13; 47,XX+16), monosomy (45, X0), tetrasomy (92,XXXX) and one case of partial deletion (46,XX,del(18)(q21). Mean methylation values of cells with apparent normal chromosome count ranged from 0–19% and 4–15% for cell lines with varying number of chromosomes (data shown in supplementary Figure 1. 3).

**RAD9A hypermethylation in tumor cell lines**

If RAD9A methylation is relevant for tumor development, and it is not due to changed chromosomal numbers, divergent methylation in tumor cell lines should be detected. We analyzed the methylation pattern in A549, MCF7, ZR–75–1, EFO27, T47D, SC263 and FaDu cell lines. The hypermethylation varied between the cell lines (mean values 11–81%, Figure 3A). The highest methylation in all three CpG’s was detected in the cell line ZR–75–1 (human breast epithelial cells) of CpG1–78%, CpG2–83%, and CpG3–83%. The methylation values seem to be independent of the RAD9A copy number. The MCF7 cell line (mean methylation 24%) has two copies of Chr.11 and one derivative (der(?)t(11;1;17;19;17)), in contrast to ZR–75–1 with no intact Chr.11 (der(11)t(11;17)x2) and FaDu cell line (mean methylation 54%) with up to three copies [51–53]. Triggered by the idea that changes in chromosomal integrity, as seen in many tumorigenic cells including EBV transformed lymphoblasts and the bone marrow samples analyzed in this study, we presumed that this might cause hypermethylation of intron 2 site in RAD9A. Therefore we generated single cell line clones from the parental FaDu cell line. Sub clonal events with gene mutations were reported by Nisar et al., [54] and recently by Ben-David and colleagues [55] with the consequence of copy number gains and losses and different drug responses. The FaDu cell line exhibits homozygous loss of function mutations in TP53 and CDKN2A genes [56], suggesting that chromosomal changes caused by the lack of proper DNA repair may be frequent. During the cultivation of the parental FaDu cell line, a certain amount of divergent cells may develop (Figure 3C). We were able to generate thirteen sub clonal cell lines with divergent RAD9A methylation patterns (Figure 3 B). Two of the sub clones, 4 and 6 exhibit high methylation values (mean methylation 75% and 73% repectively) in comparison to the parental cell line (mean methylation 54%) while others showed a lower methylation level (e.g. clone 2 and 9).
Despite different methylation levels of \textit{RAD9A}, the methylation levels remained stable during cultivation for all clones. Clone 2, 4 and 9 and the parental cell line were chosen for further characterization.

Monolayer culture growth kinetics, for the sub cell clones 2, 4, 9 and the parental FaDu cell line performed using triplicates revealed significant delayed growth for the sub clone 4 (p-value <0.0005) (Figure 3E). Clonogenic survival experiments upon irradiation done with the sub clones 2, 4, 9 and the FaDu cell line resulted in significantly reduced survival of the clone 4 in comparison to the FaDu parental cell line (adj p-value <0.0001) and clone 2 (adj p-value <0.0026) (Figure 3E). Methylation signatures for the clones in clonogenic survival experiments matched the untreated clone signatures.

\textbf{Genomic copy number typing (SNP array CGH) of the clones}

Microarray-based comparative genomic hybridization (SNP array, CGH) provides a more detailed view on chromosomal alterations. Using this technique we analyzed the clones with hypermethylation (4 and 6) in comparison to low methylation (2 and 9) and the parental FaDu cell line. No structural chromosomal differences between the analyzed sub clones could be detected; instead we identified a homozygous deletion in 15q26.1q26.2 unique to the clone 4 and a heterozygous deletion in Xq25 in clone 6. Downstream analysis using PCR and Sanger sequencing confirmed the homozygous deletion of the \textit{CHD2} and \textit{SPATA8} genes in clone 4 and the stop codon mutation c.537 538insG, p.P182fs*18 in the remaining allele of \textit{SMARCA1 (SNF2L)} gene in clone 6. Both genes harbor a helicase domain and are involved in DNA-repair and transcription regulation [57, 58] (Figure 3 D).

In order to elucidate the connection between loss of function of those important genes and hypermethylation of \textit{RAD9A} we examined two fibroblast cell lines known to have homozygous mutations either in \textit{BRCA2 (FANCD1)} or in \textit{SLX4 (FANCP1)} in contrast to normal primary fibroblast cell lines (N = 20). As expected, control cell lines exhibited mean methylation values ranging from 3-11%, in contrast to \textit{FANCD1} (mean 28%) and \textit{FANCP1} (mean 47%).

\textbf{Discussion}

The vast majority of childhood cancers occurs sporadically and cannot be explained by inherited
mutations in known tumor susceptibility genes [12]. Therefore, it seems plausible to assume that stochastic or adverse exposure events during early (intrauterine and postnatal) development increase cancer susceptibility through epigenetic reprogramming [16, 17]. Consistent with the developmental programming of cancer hypothesis, we previously identified a monozygotic twin pair discordant for childhood cancer with a constitutive BRCA1 epimutation in the twin with cancer [23]. Here, we performed a DNA methylation screen for APC, CDKN2A, EFNA5, RAD9A, and TP53 in primary fibroblasts of 40 childhood cancer patients, 20 with one primary cancer and 20 with a second primary cancer. We detected significant changes in methylation values in APC, CDKN2A and TP53 gene, suggesting future analyses in larger cohorts to confirm the described findings. The aberrant allele methylation in RAD9A, supported by additive experiments, suggests an involvement of this epigenetic change in cancer development and likely sensitivity to radiation.

We identified 5 patients with 10% mean RAD9A methylation and 2% hypermethylated alleles. Collectively, our results suggest that a considerable percentage of childhood cancer patients may have constitutive RAD9A epimutations in 4% of their normal body cells. Twenty cancer free controls had a lower frequency of RAD9A hypermethylation. Patient 1N20, who suffered from sporadic unilateral retinoblastoma, exhibited almost 12% fully methylated RAD9A alleles in fibroblasts, indicating that almost one eighth of his normal cells are endowed with epimutated RAD9A alleles.

Most retinoblastomas, which are derived from the cone photoreceptor lineage, show biallelic inactivation of the RB1 tumor suppressor gene, but additional (epi)genetic changes are likely required for tumor development [59, 60]. A small subset (1–2%) of unilateral tumors without RB1 mutations are characterized by high-level amplification of the MYCN oncogene [61]. We did not detect a RB1 germ line mutation in our retinoblastoma patient, however the mutational status of the tumor is not known. The unusually high rate of abnormally methylated RAD9A alleles in our patient indicates that RAD9A epimutations can contribute to retinoblastoma development. The retinoblastoma was cured solely by excision of the tumor, therefore no therapy related changes may be expected. Nevertheless, other patients with elevated RAD9A methylation received radiotherapy and chemotherapy.

Our irradiation experiments show that induction of DNA damage does not increase RAD9A
methylation in intron 2, both in normal fibroblasts with low \textit{RAD9A} methylation levels, and in FaDu tumor cells with \textit{RAD9A} hypermethylation. Intercalating drugs used in chemotherapy like daunorubicin also did not change the methylation of \textit{RAD9A} in our experimental setting.

It has been previously shown that hypermethylation of \textit{RAD9A} intron 2 is associated with mRNA and protein overexpression, which may be a critical step in the development of prostate, breast and other cancers [39, 62]. According to the Cancer Genome Atlas/TGCA (https://cancergenome.nih.gov/), \textit{RAD9A} is overexpressed in a wide variety of tumors, supporting an oncogenic function. EBV transformation of resting B cells to proliferating lymphoblasts induced dramatic variation in \textit{RAD9A} methylation, indicating that epigenetic dysregulation of \textit{RAD9A} may occur during malignant transformation early in tumorigenesis.

The methylation-sensitive region in intron 2 is endowed with three regulatory elements, annotated in ORegAnno (http://www.oreganno.org/). Element OREG1137234 is a binding side for the transcription factor \textit{ZNF263}, which functions as transcriptional repressor. It is plausible to assume that intron 2 hypermethylation interferes with \textit{ZNF263} binding, which then activates \textit{RAD9A} expression. The \textit{RAD9A-HUS1-RAD1} complex is recruited to sites of DNA damage and required for cell cycle arrest and DNA damage repair [63]. When translocated to mitochondria, \textit{RAD9A} binds and neutralizes the anti-apoptotic activity of BCL-2 and BCL-xL proteins, thus promoting cell death [64, 65]. However, permanent \textit{RAD9A} overexpression may also have harmful, tumor-promoting effects.

Experiments performed with EBV transformed lymphoblasts, bone marrow from leukemia patients, sub clones of the FaDu tumor cell line and analysis of Fanconi fibroblasts indicate a connection between chromosomal integrity, pathogenic alteration of crucial genes and hypermethylation of the \textit{RAD9A} locus. Analysis of CVS samples with chromosomal mis segregations indicated no relationship between methylation changes and intact chromosomes differing in numbers.

We propose that \textit{RAD9A} methylation is an early (either stochastic or environmentally induced) event, which may lead to malignant transformation in normal body cells. This in turn could affect downstream genes that are involved in further deregulation during tumor development. Constitutive epimutations (allele methylation errors) which arise in early development are likely to be present in a
mosaic state in different normal tissues of an individual. Although here we only analyzed primary skin fibroblasts, we assume that the identified RAD9A epimutations were also present in other tissues, particularly in those, which developed a tumor. Previously, we have shown that epimutations in BRCA1 and RAD51 can originate in single precursor cells [7, 8]. It is difficult to define a threshold for constitutive epimutations in normal tissues that can be associated with tumor formation. In our experience with TS epimutation screening >800 individuals [7, 8], mean methylation values of 10% and allele methylation errors of 2–3% (depending on the gene and assay) are outside the normal methylation variation range (depending on the tissue origin analyzed). The error rate for copying DNA methylation patterns during DNA replication is estimated to be 10–100 times higher than for non-replicating DNA [22, 66]. Therefore, rapidly dividing cells, i.e. stem cells during embryonal development and organogenesis, may be particularly vulnerable to acquiring methylation defects [67, 68]. Although mutations and/or epimutations in a single cell may be potentially harmful, the body must be able to cope with a (very) small proportion of cells, which need to be corrected or eliminated. Nevertheless, the risk for malignant transformations may increase with the percentage of cells with compromised genomic caretakers. When the analyzed childhood cancer patients were recruited for this study, they all had survived an initial cancer treatment (in most cases including radiation therapy) and had been tumor-free for several years. Although we did not find evidence for irradiation or chemotherapy associated RAD9A epimutations, we cannot completely exclude the possibility that the observed RAD9A hypermethylation in some patients is a consequence of tumor treatment which leads to mutation of important genes. We propose that the constitutive RAD9A epimutations arose early in development leading to a predisposition of the compromised cells to tumorigenesis. Larger prospective studies are needed to correlate constitutive epimutations in RAD9A and other susceptibility genes with life-long cancer risk.

Conclusion
This study revealed elevated mean methylation levels of RAD9A intron 2 in primary fibroblasts of former childhood cancer patients. Deep bisulfite sequencing of RAD9A in these patients displayed elevated allelic methylation errors, which must be considered as functionally relevant epimutations.
We propose that the constitutive RAD9A epimutations arose early in development, predisposing the compromised cells to tumorigenesis and patients to increased childhood cancer risk. Analyses of tumor cell clones with high methylation levels of RAD9A suggest a connection between methylation levels of the RAD9A intron 2 locus and a homozygous inactivation of important genes with followed effects on carcinogenesis and progression. Larger prospective studies are needed to correlate constitutive epimutations in RAD9A and other susceptibility genes with life-long cancer risk and progression of carcinogenesis.

Abbreviations
TS—tumor suppressor, HNPCC—hereditary non-polyposis colon cancer, NF1—neurofibromatosis type 1, CS—Costello syndrome, CFCS—cardiofaciocutaneous syndrome, EBV—Epstein Barr virus, B-ALL—acute lymphocytic leukemia, AML—Acute myeloid leukemia, PBL—plasmablastic lymphoma, FACS—flow cytometric cell cycle, DMEM—Dulbecco’s modified Eagle’s medium, FBS—foetal bovine serum, EMRs—epimutation rates, DBS—deep bisulfite sequencing, Gy—Gray, RA—refractory anemia. CVS—chorionic villus sampling.

Declarations
Ethics approval and consent to participate and for publication
This study was approved by the Ethics Committee of the Medical Association of Rhineland-Palatinate (no. 837.440.03 [4102], 837.103.04 (4261) and no. 837.262.12(8363-F]).

Consent for publication
Written informed consent to use fibroblasts for research purposes was obtained after genetic counselling from all participating patients.

Disclosure of potential conflicts of interest
The authors declare no conflict of interest

Funding
This study was supported by BMBF German ministry for education and science. Grants: 02NUK016A and 02NUK042A
Author contributions

D.G. and J.B contributed equally to this work, and should be considered co-first authors, MD analyzed the bNGS data. DG, OS, ML, JM prepared the cell culture radiation and pyro sequencing experiments. HR helped with the analysis of pyro sequencing data. AP performed the statistical analysis of the pyro sequencing data, colony formation assay and the growth kinetics. JB and OS prepared the bisulfite sequence bNGS, and provided the data interpretation. IS prepared the replicative senescence experiment, including data analysis and graphic. ML made the array analysis of FaDu and sub clones, MM designed as the principal investigator the recruitment of cancer free controls (KiKme - childhood cancer study), CS, PS-K and MM organized the patient’s recruitment. DG, TH, DP and HS conceived and designed the study and wrote the paper.

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Acknowledgment

First of all, we would like to thank the patients for participating in this study. Our clinical staff also deserves special thanks. We also thank Ursula Dique-Kaiser, Britta Weber, Danuta Wieczorek, Martina Mihm and Martina Hermanss for technical support. We kindly thank Prof. Detlev Schindler for the donation of Fanconi Fibroblasts. We are grateful to Benjamin Irmscher for providing the daunorubicin samples. Special thanks to Heather Chorzempa for the proof reading of this manuscript.

References

[1] Dumont N, Arteaga CL. Transforming growth factor-beta and breast cancer: Tumor promoting effects of transforming growth factor-β. Breast Cancer Res 2000; 2(2): 93
[https://doi.org/10.1186/bcr44]

[2] Karakosta A, Golas C, Charalabopoulos A, Peschos D, Batistatou A, Charalabopoulos K. Genetic models of human cancer as a multistep process. Paradigm models of colorectal cancer, breast cancer, and chronic myelogenous and acute lymphoblastic leukaemia. J Exp Clin Cancer Res 2005; 24(4):
505–14

[PMID: 16471312]

[3] Ehrlich M. Cancer-linked DNA hypomethylation and its relationship to hypermethylation. Curr Top Microbiol Immunol 2006; 310: 251–74

[PMID: 16909914]

[4] Ting AH, McGarvey KM, Baylin SB. The cancer epigenome--components and functional correlates. Genes Dev 2006; 20(23): 3215–31

[https://doi.org/10.1101/gad.1464906][PMID: 17158741]

[5] Chan TL, Yuen ST, Kong CK, et al. Heritable germline epimutation of MSH2 in a family with hereditary nonpolyposis colorectal cancer. Nat Genet 2006; 38(10): 1178–83

[https://doi.org/10.1038/ng1866][PMID: 16951683]

[6] Goel A, Nguyen T-P, Leung H-CE, et al. De novo constitutional MLH1 epimutations confer early-onset colorectal cancer in two new sporadic Lynch syndrome cases, with derivation of the epimutation on the paternal allele in one. Int J Cancer 2011; 128(4): 869–78

[https://doi.org/10.1002/ijc.25422][PMID: 20473912]

[7] Hansmann T, Pliushch G, Leubner M, et al. Constitutive promoter methylation of BRCA1 and RAD51C in patients with familial ovarian cancer and early-onset sporadic breast cancer. Hum Mol Genet 2012; 21(21): 4669–79

[https://doi.org/10.1093/hmg/ddr308][PMID: 22843497]

[8] Böck J, Appenzeller S, Haertle L, et al. Single CpG hypermethylation, allele methylation errors, and decreased expression of multiple tumor suppressor genes in normal body cells of mutation-negative early-onset and high-risk breast cancer patients. Int J Cancer 2018; 143(6): 1416–25

[https://doi.org/10.1002/ijc.31526][PMID: 29659014]

[9] Hitchins MP. Finding the needle in a haystack: identification of cases of Lynch syndrome with MLH1 epimutation. Fam Cancer 2016; 15(3): 413–22

[https://doi.org/10.1007/s10689-016-9887-3][PMID: 26886015]

[10] Ligtenberg MJL, Kuiper RP, Chan TL, et al. Heritable somatic methylation and inactivation of MSH2
in families with Lynch syndrome due to deletion of the 3' exons of TACSTD1. Nat Genet 2009; 41(1): 112–7
[https://doi.org/10.1038/ng.283][PMID: 19098912]

[11] Raval A, Tanner SM, Byrd JC, et al. Downregulation of death-associated protein kinase 1 (DAPK1) in chronic lymphocytic leukemia. Cell 2007; 129(5): 879–90
[https://doi.org/10.1016/j.cell.2007.03.043][PMID: 17540169]

[12] D'Orazio JA. Inherited cancer syndromes in children and young adults. J Pediatr Hematol Oncol 2010; 32(3): 195–228
[https://doi.org/10.1097/MPH.0b013e3181ced34c][PMID: 20186103]

[13] van Echten-Arends J, Mastenbroek S, Sikkema-Raddatz B, et al. Chromosomal mosaicism in human preimplantation embryos: a systematic review. Hum Reprod Update 2011; 17(5): 620–7
[https://doi.org/10.1093/humupd/dmr014][PMID: 21531753]

[14] Hafner C, Groesser L. Mosaic RASopathies. Cell Cycle 2013; 12(1): 43–50
[https://doi.org/10.4161/cc.23108][PMID: 23255105]

[15] Kratz CP, Rapisuwon S, Reed H, Hasle H, Rosenberg PS. Cancer in Noonan, Costello, cardiofaciocutaneous and LEOPARD syndromes. Am J Med Genet C Semin Med Genet 2011; 157C(2): 83–9
[https://doi.org/10.1002/ajmg.c.30300][PMID: 21500339]

[16] Walker CL, Ho S-m. Developmental reprogramming of cancer susceptibility. Nat Rev Cancer 2012; 12(7): 479–86
[https://doi.org/10.1038/nrc3220][PMID: 22695395]

[17] Marshall GM, Carter DR, Cheung BB, et al. The prenatal origins of cancer. Nat Rev Cancer 2014; 14(4): 277–89
[https://doi.org/10.1038/nrc3679][PMID: 24599217]

[18] Gale KB, Ford AM, Repp R, et al. Backtracking leukemia to birth: Identification of clonotypic gene fusion sequences in neonatal blood spots. Proc Natl Acad Sci U S A 1997; 94(25): 13950–4
[PMID: 9391133]
[19] Hjalgrim LL, Madsen HO, Melbye M, et al. Presence of clone-specific markers at birth in children with acute lymphoblastic leukaemia. Br J Cancer 2002; 87(9): 994–9
[https://doi.org/10.1038/sj.bjc.6600601][PMID: 12434291]

[20] McHale CM, Wiemels JL, Zhang L, et al. Prenatal origin of childhood acute myeloid leukemias harboring chromosomal rearrangements t(15;17) and inv(16). Blood 2003; 101(11): 4640–1
[https://doi.org/10.1182/blood-2003-01-0313][PMID: 12756163]

[21] Hitzler JK, Zipursky A. Origins of leukaemia in children with Down syndrome. Nat Rev Cancer 2005; 5(1): 11-20
[https://doi.org/10.1038/nrc1525][PMID: 15630411]

[22] Bennett-Baker PE, Wilkowski J, Burke DT. Age-associated activation of epigenetically repressed genes in the mouse. Genetics 2003; 165(4): 2055–62
[PMID: 14704185]

[23] Galetzka D, Hansmann T, El Hajj N, et al. Monozygotic twins discordant for constitutive BRCA1 promoter methylation, childhood cancer and secondary cancer. Epigenetics 2012; 7(1): 47–54
[https://doi.org/10.4161/epi.7.1.18814][PMID: 22207351]

[24] Magnusson S, Borg A, Kristoffersson U, Nilbert M, Wiebe T, Olsson H. Higher occurrence of childhood cancer in families with germline mutations in BRCA2, MMR and CDKN2A genes. Fam Cancer 2008; 7(4): 331–7
[https://doi.org/10.1007/s10689-008-9195-7][PMID: 18481196]

[25] Sharma B, Preet Kaur R, Raut S, Munshi A. BRCA1 mutation spectrum, functions, and therapeutic strategies: The story so far. Curr Probl Cancer 2018; 42(2): 189–207
[https://doi.org/10.1016/j.crrproblcancer.2018.01.001][PMID: 29452958]

[26] Sedic M, Skibinski A, Brown N, et al. Haploinsufficiency for BRCA1 leads to cell-type-specific genomic instability and premature senescence. Nat Commun 2015; 6: 7505
[https://doi.org/10.1038/ncomms8505][PMID: 26106036]

[27] Shim YH1 et al., 2000. Correlation of p16 hypermethylation with p16 protein loss in sporadic gastric carcinomas. Lab Invest; 2000(May: 80(5)): 689–95.
[28] Esteller M, Fraga MF, Guo M, et al. DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. Hum Mol Genet 2001; 10(26): 3001–7
[PMID: 11751682]

[29] Sherr CJ, Roberts JM. Living with or without cyclins and cyclin-dependent kinases. Genes Dev 2004; 18(22): 2699–711
[https://doi.org/10.1101/gad.1256504][PMID: 15545627]

[30] Nieuwenhuis MH, Vasen HFA. Correlations between mutation site in APC and phenotype of familial adenomatous polyposis (FAP): a review of the literature. Crit Rev Oncol Hematol 2007; 61(2): 153–61
[https://doi.org/10.1016/j.critrevonc.2006.07.004][PMID: 17064931]

[31] Bienz M, Hamada F. Adenomatous polyposis coli proteins and cell adhesion. Curr Opin Cell Biol 2004; 16(5): 528–35
[https://doi.org/10.1016/j.ceb.2004.08.001][PMID: 15363803]

[32] Caldwell CM, Kaplan KB. The role of APC in mitosis and in chromosome instability. Adv Exp Med Biol 2009; 656: 51–64
[PMID: 19928352]

[33] Kuang S-Q, Tong W-G, Yang H, et al. Genome-wide identification of aberrantly methylated promoter associated CpG islands in acute lymphocytic leukemia. Leukemia 2008; 22(8): 1529–38
[https://doi.org/10.1038/leu.2008.130][PMID: 18528427]

[34] Shi H, Guo J, Duff DJ, et al. Discovery of novel epigenetic markers in non-Hodgkin's lymphoma. Carcinogenesis 2007; 28(1): 60–70
[https://doi.org/10.1093/carcin/bgl092][PMID: 16774933]

[35] Olivier M, Hollstein M, Hainaut P. TP53 mutations in human cancers: origins, consequences, and clinical use. Cold Spring Harb Perspect Biol 2010; 2(1): a001008
[https://doi.org/10.1101/cshperspect.a001008][PMID: 20182602]

[36] Shirole NH, Pal D, Kastenhuber ER, et al. TP53 exon-6 truncating mutations produce separation of function isoforms with pro-tumorigenic functions. Elife 2016; 5
[37] Lieberman HB, Bernstock JD, Broustas CG, Hopkins KM, Leloup C, Zhu A. The role of RAD9 in tumorigenesis. J Mol Cell Biol 2011; 3(1): 39–43
[https://doi.org/10.1093/jmcb/mjq039][PMID: 21278450]

[38] Cheng CK, Chow LWC, Loo WTY, Chan TK, Chan V. The cell cycle checkpoint gene Rad9 is a novel oncogene activated by 11q13 amplification and DNA methylation in breast cancer. Cancer Res 2005; 65(19): 8646–54
[https://doi.org/10.1158/0008-5472.CAN-04-4243][PMID: 16204032]

[39] Zhu A, Zhang CX, Lieberman HB. Rad9 has a functional role in human prostate carcinogenesis. Cancer Res 2008; 68(5): 1267–74
[https://doi.org/10.1158/0008-5472.CAN-07-2304][PMID: 18316588]

[40] Weis E, Schoen H, Victor A, et al. Reduced mRNA and protein expression of the genomic caretaker RAD9A in primary fibroblasts of individuals with childhood and independent second cancer. PLoS ONE 2011; 6(10): e25750
[https://doi.org/10.1371/journal.pone.0025750][PMID: 21991345]

[41] Magadier F, Billard Lise-Marie, Wittmann, Gaelle, Frappart Lucien, et al. Regional methylation of the 5’ end CpG island of BRCA1 is associated with reduced gene expression in human somatic cells. The FASEB Journal 2000; 14(11): 1585–94
[https://doi.org/10.1096/fj.99-0817com]

[42] Weber M, Hellmann I, Stadler MB, et al. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet 2007; 39(4): 457–66
[https://doi.org/10.1038/ng1990][PMID: 17334365]

[43] Phipps SMO, Berletch JB, Andrews LG, Tollefsbol TO. Aging cell culture: methods and observations. Methods Mol Biol 2007; 371: 9–19
[https://doi.org/10.1007/978-1-59745-361-5_2][PMID: 17634570]

[44] Flunkert J, Maierhofer A, Dittrich M, et al. Genetic and epigenetic changes in clonal descendants of irradiated human fibroblasts. Exp Cell Res 2018; 370(2): 322–32
[45] Maierhofer A, Flunkert J, Dittrich M, et al. Analysis of global DNA methylation changes in primary human fibroblasts in the early phase following X-ray irradiation. PLoS ONE 2017; 12(5): e0177442

[46] Zunino F, Gambetta R, Di Marco A, Zaccara A. Interaction of daunomycin and its derivatives with DNA. Biochimica et Biophysica Acta (BBA) - Nucleic Acids and Protein Synthesis 1972; 277(3): 489–98

[47] Peterson C, Trouet A. Transport and storage of daunorubicin and doxorubicin in cultured fibroblasts. Cancer Res 1978; 38(12): 4645–9

[48] Przybylska M, Koceva-Chyla A, Rózga B, Józwiak Z. Cytotoxicity of daunorubicin in trisomic (+21) human fibroblasts: relation to drug uptake and cell membrane fluidity. Cell Biol Int 2001; 25(2): 157-70

[49] Neitzel H. A routine method for the establishment of permanent growing lymphoblastoid cell lines. Hum Genet 1986; 73(4): 320–6

[50] Hernandez-Vargas H, Gruffat H, Cros MP, et al. Viral driven epigenetic events alter the expression of cancer-related genes in Epstein-Barr-virus naturally infected Burkitt lymphoma cell lines. Sci Rep 2017; 7(1): 5852

[51] Davidson JM, Gorringe KL, Chin S-F, et al. Molecular cytogenetic analysis of breast cancer cell lines. Br J Cancer 2000; 83(10): 1309–17

[52] Rangan S. A new human cell line (FaDu) from a hypopharyngeal carcinoma.

[53] Kytl S, Rummukainen J, Nordgren A, et al. Chromosomal alterations in 15 breast cancer cell lines by comparative genomic hybridization and spectral karyotyping. Genes Chromosom. Cancer 2000;
[54] Amin NA, Seymour E, Saiya-Cork K, Parkin B, Shedden K, Malek SN. A Quantitative Analysis of Subclonal and Clonal Gene Mutations before and after Therapy in Chronic Lymphocytic Leukemia. Clin Cancer Res 2016; 22(17): 4525–35

[55] Ben-David U, Siranosian B, Ha G, et al. Genetic and transcriptional evolution alters cancer cell line drug response. Nature 2018; 560(7718): 325–30

[56] Nichols AC, Yoo J, Palma DA, et al. Frequent mutations in TP53 and CDKN2A found by next-generation sequencing of head and neck cancer cell lines. Arch Otolaryngol Head Neck Surg 2012; 138(8): 732–9

[57] Luijsterburg MS, Krijger I de, Wiegant WW, et al. PARP1 Links CHD2-Mediated Chromatin Expansion and H3.3 Deposition to DNA Repair by Non-homologous End-Joining. Mol Cell 2016; 61(4): 547–62

[58] Eckey M, Kuphal S, Straub T, et al. Nucleosome remodeler SNF2L suppresses cell proliferation and migration and attenuates Wnt signaling. Molecular and Cellular Biology 2012; 32(13): 2359–71

[59] Dimaras H, Khetan V, Halliday W, et al. Loss of RB1 induces non-proliferative retinoma: increasing genomic instability correlates with progression to retinoblastoma. Hum Mol Genet 2008; 17(10): 1363–72

[60] Thériault BL, Dimaras H, Gallie BL, Corson TW. The genomic landscape of retinoblastoma: a review. Clin Experiment Ophthalmol 2014; 42(1): 33–52
[61] Rushlow DE, Mol BM, Kennett JY, et al. Characterisation of retinoblastomas without RB1 mutations: genomic, gene expression, and clinical studies. The Lancet Oncology 2013; 14(4): 327–34 [https://doi.org/10.1016/S1470-2045(13)70045-7]

[62] Broustas CG, Zhu A, Lieberman HB. Rad9 protein contributes to prostate tumor progression by promoting cell migration and anoikis resistance. J Biol Chem 2012; 287(49): 41324–33 [https://doi.org/10.1074/jbc.M112.402784][PMID: 23066031]

[63] Tsai F-L, Kai M. The checkpoint clamp protein Rad9 facilitates DNA-end resection and prevents alternative non-homologous end joining. Cell Cycle 2014; 13(21): 3460–4 [https://doi.org/10.4161/15384101.2014.958386][PMID: 25485590]

[64] Komatsu K, Miyashita T, Hang H, et al. Human homologue of S. pombe Rad9 interacts with BCL-2/BCL-xL and promotes apoptosis. Nat Cell Biol 2000; 2(1): 1–6 [https://doi.org/10.1038/71316][PMID: 10620799]

[65] Yoshida K, Komatsu K, Wang H-G, Kufe D. c-Abl Tyrosine Kinase Regulates the Human Rad9 Checkpoint Protein in Response to DNA Damage. Molecular and Cellular Biology 2002; 22(10): 3292–300 [https://doi.org/10.1128/MCB.22.10.3292-3300.2002]

[66] Horsthemke B. Epimutations in human disease. Curr Top Microbiol Immunol 2006; 310: 45–59 [PMID: 16909906]

[67] Tomasetti C, Li L, Vogelstein B. Stem cell divisions, somatic mutations, cancer etiology, and cancer prevention. Science 2017; 355(6331): 1330–4 [https://doi.org/10.1126/science.aaf9011][PMID: 28336671]

[68] Lopez-Lazaro M. The stem cell division theory of cancer. Crit Rev Oncol Hematol 2018; 123: 95–113 [https://doi.org/10.1016/j.critrevonc.2018.01.010][PMID: 29482784]

[69] Manuela Marron. Cancer in childhood and molecular epidemiology - The KIKME case-control study [abstract] 2017: 77 (13 Suppl): Abstract nr 4261. doi:10.1158/1538-7445.AM2017-4261.

[70] Victor A, Weis E, Messow CM, et al. Searching for gene expression differences in primary
fibroblasts between patients with one and two neoplasms in childhood. Pediatr Hematol Oncol 2013; 30(1): 33–45
[https://doi.org/10.3109/08880018.2012.735747][PMID: 23140311]

[71] Weis E, Galetzka D, Herlyn H, Schneider E, Haaf T. Humans and chimpanzees differ in their cellular response to DNA damage and non-coding sequence elements of DNA repair-associated genes. Cytogenet Genome Res 2008; 122(2): 92–102
[https://doi.org/10.1159/000163086][PMID: 19096204]

[72] Menegakis A, Yaromina A, Eicheler W, et al. Prediction of clonogenic cell survival curves based on the number of residual DNA double strand breaks measured by gammaH2AX staining. Int J Radiat Biol 2009; 85(11): 1032–41
[https://doi.org/10.3109/09553000903242149][PMID: 19895280]

[73] Rahmann S, Beygo J, Kanber D, Martin M, Horsthemke B, Buiting K. Amplikyzer: Automated methylation analysis of amplicons from bisulfite flowgram sequencing 2013.

[74] R Core Team (2015). R: A language and environment for statistical computing. Vienna, Austria. URL https://www.R-project.org/.

[75] Hughes S, Jones JL. The use of multiple displacement amplified DNA as a control for methylation specific PCR, pyrosequencing, bisulfite sequencing and methylation-sensitive restriction enzyme PCR. BMC Mol Biol 2007; 8: 91
[https://doi.org/10.1186/1471-2199-8-91][PMID: 17939862]

[76] Feng W, Shen L, Wen S, et al. Correlation between CpG methylation profiles and hormone receptor status in breast cancers. Breast Cancer Res 2007; 9(4): R57
[https://doi.org/10.1186/bcr1762][PMID: 17764565]

[77] Schatz P, Distler J, Berlin K, Schuster M. Novel method for high throughput DNA methylation marker evaluation using PNA-probe library hybridization and MALDI-TOF detection. Nucleic Acids Res 2006; 34(8): e59
[https://doi.org/10.1093/nar/gkl218][PMID: 16670426]

[78] Flanagan JM, Popendikyte V, Pozdniakovaite N, et al. Intra- and Interindividual Epigenetic
Variation in Human Germ Cells. Am J Hum Genet 2006; 79(1): 67–84

[PMID: 16773567]

Tables

Table 1. Results of methylation analysis (by bisulfite pyrosequencing and DBS) of patients with suspected e

| Sample ID | First cancer* | Second cancer* | Gene | Mean (%) of all 1N and 2N patients | Mean (%) of the given patient |
|-----------|---------------|---------------|------|-----------------------------------|------------------------------|
| 1N08      | ALL           | -             | APC  | 5                                 | 9                            |
| 1N15      | ALL           | -             | CDKN2A | 3                            | 6.5                          |
| 2N12      | Rhabdomyosarcoma | Liver cancer | TP53 | 97                               | 90                           |
| 1N06 (control) | ALL         | -             | RAD9A | 7                                | 0%                           |
| 1N04      | Hodgkin lymphoma | -         | RAD9A | 7                                | 13                           |
| 1N07      | ALL           | -             | RAD9A | 7                                | 10                           |
| 1N14      | Hodgkin lymphoma | -         | RAD9A | 7                                | 14                           |
| 1N20      | Retinoblastoma | -             | RAD9A | 7                                | 31                           |
| 2N21      | ALL           | RA            | RAD9A | 7                                | 12                           |

* ALL, acute lymphoblastic leukemia; RA, refractory anemia

Table 2. Effects of irradiation on RAD9A methylation
| Cells   | Single dose       | Mean RAD9A methylation | Cells   | Serial doses | Mean RAD. methylation |
|---------|-------------------|------------------------|---------|--------------|-----------------------|
| 0N18    | 15 min after 0 Gy | 8%                     | 1N08    | 0 Gy         | 5                     |
| "       | 2 h after 0 Gy    | 7%                     | "       | 8x 2 Gy      | 5                     |
| "       | 24 h after 0 Gy   | 7%                     | "       | 4x 4 Gy      | 5                     |
| "       | 15 min after 2 Gy | 9%                     | "       | 8x 4 Gy      | 5                     |
| "       | 2 h after 2 Gy    | 7%                     | "       | 8x 4 Gy      | 5                     |
| "       | 24 h after 2 Gy   | 8%                     | 0N12    | 0 Gy         | 9                     |
| "       | 15 min after 5 Gy | 7%                     | "       | 10x 4 Gy     | 8                     |
| "       | 2 h after 5 Gy    | 9%                     | "       | 10x 4 Gy     |                       |
| "       | 24 h after 5 Gy   | 7%                     | 2N12    | 0 Gy         | 5                     |
| "       | 15 min after 8 Gy | 7%                     | "       | 10x 2 Gy     | 5                     |
| "       | 2 h after 8 Gy    | 9%                     | "       | 10x 2 Gy     |                       |
| "       | 2 h after 8 Gy    | 7%                     |         |              |                       |

**Figures**
Figure 1

Methylation analysis of candidate genes. Box plots showing the distribution of (A) APC, (B) CDKN2A, (C) EFNA, (D) RAD9A, and (E) TP53 methylation values in 20 healthy controls (0N), 20 one-cancer (1N), and 20 two-cancer (2N) patients including outliers which are listed in Table 1. The bottom and the top of the boxes represent the 25th and 75th percentiles, respectively. The median is represented by a vertical dotted line. Bars extend from the boxes to at most 1.5 times the height of the box. Statistical comparison was performed using Kruskal-Wallis rank sum test.
Figure 2

Growth kinetics and methylation of RAD9A in five independent fibroblast cell lines. (A) Growth curves of fibroblast strains from controls 0N01, 0N02, 0N03, 0N24 and cancer patient 2N24 until senescence. The x-axis indicates the culture time and the y-axis the number of population doublings. (B-F) Distribution of the APC, BRCA1, CDKN2A, RAD9A, and TP53 methylation values of the 5 analyzed strains at passages 7, 17, and 25. The bottom and the top of the boxes represent the 25th and 75th percentiles, respectively. The median is represented by a vertical line. Bars extend from the boxes to at most 1.5 times the height of the box.
Figure 3

Methylation values of intron 2 site of RAD9A in tumor cell lines and FaDu sub clones. (A) Mean methylation values in A549, MCF7, ZR-75, EFO27, T47D, FaDu and SC263 cell lines. (B) Mean methylation of FaDu sub clones. Highest values were determined in clone 4 and clone 6. (C) Crystal violet staining of an exemplary FaDu colony. Divergent cells are depicted by arrows. (D) Mutation analysis in clone 4 and clone 6. CHD2 and SPATA8 are homozygous deleted in clone 4. Loss of function is shown for SMARCA1 in clone 6. (E) The graphs show cumulative populations doublings for sub clones 2, 4, 9 and parental FaDu cell line and survival post radiation for the sub clone 4 and FaDu. Congenic survival was calculated as percentage of the non-irradiated controls and is shown as means + s.d. of 3 independent experiments. Linear quadratic fitting was performed, and results were compared using F-testing.
Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

suptables.docx
Supplementary Fig.1. 1-3 27.06.19.tif