Patient-derived organoid model for prediction of cancer-risk in patient with germline mutation of mismatch repair genes

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Patient-derived organoid model for prediction of cancer-risk in patient with germline mutation of mismatch repair genes

Directed by Professor Tae Il Kim

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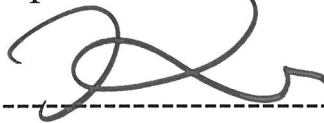Master of Medical Science

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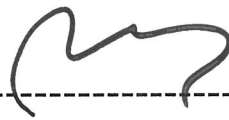
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ABSTRACT

**Patient-derived organoid model for prediction of cancer-risk in patient
with germline mutation of mismatch repair genes**

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About 10~15% of Colorectal cancer (CRC) shows MSI-tumor, and about 20% of MSI-tumor is caused by germline mutation of Mismatch repair (MMR) genes MLH1, MSH2, MSH6, PMS2, and EPCAM, which is known as Lynch syndrome (LS) or Hereditary Non-polyposis Colorectal Cancer Syndrome (HNPCC).

Although Lynch syndrome patients have a high risk of colorectal cancer, not all of them have the same risk of developing CRC even in the same pathogenic germline mutations of MMR gene. We suggest the individualized prediction model for CRC risk using Lynch syndrome patient-derived organoid (PDO).

First of all, we measured organoid response to the cytotoxic effect of a methylating agent, N-Methyl-N'-Nitro-N-Nitrosoguanidine (MNNG), based on the DNA damage induced apoptosis. We treated colon organoids with various dose and period of MNNG. However, we could not find significantly rapid change of normal organoid growth. Then, to increase the effect of MNNG with induction of apoptosis in colon organoids, we additionally treated O^6BG, MGMT inhibitor, and ATR inhibitor. We found that the cytotoxic effect by combined treatment of MNNG, O^6BG and ATR inhibitor in normal organoids were higher than MMR gene mutated organoids, which was detected after several passage of organoids. Next, for early detection of differential change between normal organoids and LS-PDOs, we examined DNA damage response by analyzing the expression of γH2AX, which is a DNA damage recognition marker for detecting in earlier phase of DNA damage. Then, after treatment of MNNG and O^6BG we found a higher expression of γH2AX in normal

organoids than in the MMR gene mutated organoids.

To identify the mutations that accumulated by MNNG, we performed the analysis of the Sanger Sequence and Whole Genome Sequence (WGS). We treated MNNG in colon organoids and harvest with several recovery times. We found that normal organoids showed decreased base transitions over time, however, LS-PDOs showed increased base transitions over time. In addition, according to analysis of WGS data, MNNG-induced mutation number was significantly increased in MLH1 gene mutated organoids, compared to normal organoids.

In conclusion, the measurement of DNA damage response by MNNG and O^6BG in Lynch syndrome patient-derived organoid model could serve as an individualized prediction model of CRC risk in Lynch syndrome patients.

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I. INTRODUCTION

Colorectal cancer (CRC) is the third most common cause of cancer-related deaths worldwide.[1] Approximately 65% of CRC cases are sporadic with no family history or apparent genetic predisposition. The majority of sporadic CRCs (~85%) show chromosomal instability (CIN), with changes in chromosome number or structure. These changes involve the gain or loss of chromosome segments and the loss of heterozygosity (LOH), which then cause gene copy number variations (CNVs). These alterations activate the expression of tumor-related genes and the pathways essential for CRC initiation and progression. [2] The remaining cases (~15%) have high-frequency microsatellite of instability (MSI) phenotypes. Microsatellite Instability-High (MSI-H) status shows certain sections of microsatellites that have become unstable, since the major mismatch repair (MMR) genes are not functioning properly. The majority of sporadic MSI-H CRC is caused by the hypermethylated promoter of the MLH1 gene and about 20% of MSI-H tumor is from the germline mutation of the MMR genes (MLH1, MSH2, MSH6, PMS2 and EPCAM), which is known as Lynch syndrome (LS) or Hereditary Non-Polyposis Colorectal Cancer Syndrome (HNPCC). [3]

Identifying patients with Lynch syndrome is clinically important because it is the most common cause of hereditary CRC. Individuals with Lynch syndrome tend to

have fewer than ten adenomatous polyps cumulatively in their life. Adenomas are commonly seen in patients younger than the age of 40 and frequently have a villous growth pattern with moderate to high-grade dysplasia. Adenomas in individuals with Lynch syndrome tend to transform into cancers more rapidly than those in individuals of the general population.[4]

Lynch Syndrome patients have a 60–80% lifetime risk of developing CRC and other tumors, including endometrial and ovarian cancer, urologic cancer, and gastric cancer that typically develop around the fifth decade of life and are characterized by MSI-H and the loss of expression of the corresponding MMR protein MLH1, MSH2, MSH6, PMS2, or EPCAM. [3] Mutations in the MLH1 or MSH2 gene tend to lead to a higher risk (70 to 80%) of developing cancer in a person's lifetime, while mutations in the MSH6 or PMS2 gene have a relatively lower risk (25 to 60%) of cancer development.[5]

Consequently, most Lynch syndrome patients genetically carry a mutation in one allele of their MMR genes. However, 30% of lynch syndrome patients have a variant of uncertain (or unknown) significance (VUS). It is a genetic variant that has been identified through genetic testing, whose significance to an organism's function or health is not yet unknown. That means whose impact on the individual's cancer risk is not yet known.[6] Therefore, VUS represents a major clinical challenge because Lynch Syndrome diagnosis of carriers and their relatives is impossible due to lack of satisfying significance.

In addition, although Lynch syndrome patients have a high risk of colorectal cancer, not all of them have the same risk of developing CRC. However, there is no way to evaluate the difference in cancer or tumor risk among Lynch syndrome patients. Even in the same pathogenic germline mutations of the MMR gene, individualized risk of cancer development is various, suggesting the necessity of an individualized prediction model for cancer risk.

As for the functional assay of MMR genes and their variants, some models using

classical cell line and animal model in biomedical research has been successful in the research field of molecular mechanism and biologic significance.[7] However, these results could not explain the individual difference in cancer risk in Lynch syndrome. Recently, with the advent of human organoids, it has become possible to recreate the architecture and physiology of human organs in remarkable detail. Human organoids have been used to study infectious diseases, genetic disorders, and cancers through the genetic engineering of human cells. Therefore, the human organoids can provide valuable information about the mechanisms of specific diseases and a patients-specific in vitro disease model, in addition to their potential application in pharmaceutical drug testing and molecular medicine.

Therefore, we developed the patient-derived organoid model to measure the biological significance of MMR gene variants and predict cancer risks in individual Lynch syndrome patients.

II. MATERIALS AND METHODS

1. Human organoid culture and measurement of organoid growth.

Human colon organoids were derived from normal tissues of a resected colon segment of patients diagnosed with colorectal cancer and from endoscopy material of healthy Lynch syndrome patients. The tissues from patients were washed three times with PBS and cut into pieces. Subsequently, the fragments were incubated in a digestion solution (DMEM with collagenase). After incubation for 30~40 minutes at 37°C, they were pipetted to liberate the crypts. Then add PBS and centrifuge at 1,500 rpm for 3 minutes. Isolated crypts were pelleted and cultured in domes with Matrigel (Corning) then add organoid culture medium. The composition of organoid culture medium is: Basal culture medium with 50% Wnt/R-Spondin conditioned medium, 10% Noggin, B27 and N2 supplements (Gibco), n-Acetyl Cysteine (Sigma-Aldrich), Nicotinamide (Sigma-Aldrich), human EGF, Gastrin (Sigma-Aldrich), A83-01 (Sigma-Aldrich), SB202190 (Sigma-Aldrich), and Primocin (InvivoGen). Organoid culture medium was refreshed every two days. To passage the organoids, Matrigel was broken up by pipetting and organoids were collected in a tube. The organoids were centrifuged at 1,500 rpm for 3 min and the medium removed. 400μl Triple Express (Invitrogen) was added and the organoids were incubated at 37°C for approximately 5 min. Medium was added with 10 μM Y27632 (Sigma-Aldrich) for the first 2 days. The ten organoids were counted which the size was over 50μm in initial day for measurement of growth.

2. Drug treatment

The organoids were exposed to N-Methyl-N'-Nitro-N-Nitrosoguanidine (MNNG) (Tokyo Chemical Industry co.). To exclude differences in the efficacy of MNNG due to O^6-methylguanine-DNA methyltransferase (MGMT) activity, cells were exposed to 25μM O^6-Benzylguanine (O^6BG) (Sigma-Aldrich) and 10 μM ATR inhibitor (Sigma-Aldrich) was also used to induce apoptosis. After the organoids were pre-treated with O^6-benzylguanine, they were treated with 15μM and 25 μM MNNG for

45 minutes, 24 hours and 48 hours, and ATR inhibitor treated for 20 hours. All chemicals were dissolved in dimethyl sulfoxide and diluted in deionized water.

3. Immunofluorescence and DNA damage measurement in organoids

The organoid culture media was removed, and the organoid was fixed with 4% paraformaldehyde (PFA) for 30 minutes at room temperature. Then they were washed extensively at least 3 times with PBS for removal of the fixing solution. For staining, organoids were permeabilized with 5% Triton-X for 20 minutes at room temperature. The organoids were blocked with 3% BSA in PBS for 1 hour at room temperature. Then, they were incubated with rabbit anti-γH2AX primary antibody (cell signaling, #9718) overnight at 4°C in 1:500 dilution. The organoids were washed several times with PBS and incubated with secondary anti-rabbit antibodies for 1 hour at room temperature in the dark and washed again with PBS. The organoids were washed, DAPI was added and washed again with PBS. The organoids were analyzed with an LSM700 laser scanning confocal microscope (Carl Zeiss) and Operetta CLS (PerkinElmer).

4. Immunohistochemistry

The organoids stained for γH2AX was performed on 4μm sections of 4% PFA fixed, paraffin-embedded. The paraffin embedded sections were deparaffinized in xylene and rehydrated in gradually decreasing concentrations of ethanol. Antigen retrieval was performed using sodium citrated buffer (10mM, pH 6.0) in a heated pressure cooker for 5 or 7 minutes. After incubation with 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity, sections were incubated in a blocking reagent for 30 minutes at room temperature. Sections were incubated with the primary antibody overnight at 4°C, followed by the secondary antibody for 30 minutes at room temperature. After the slides had been developed with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA), immunodetection was performed using DAB solution (Dako, Carpinteria, CA, USA). After counterstaining with hematoxylin, IHC staining was evaluated by light microscopy and

immunoactivity was assessed based on the proportion of immunostaned organoids.

5. Western blot

Samples were lysed using lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40) containing protease inhibitors. Protein content was quantified using standard BCA assay (BioRad) and equal amounts of protein were run on polyacrylamide gels (15%) and transferred to PVDF membranes. After blocking with 10% skim milk in 0.1% TBST (Tween20 in TBS solution) for 60 minutes, membranes were probed with primary antibodies and incubated at 4°C overnight. Bound antibodies were detected with secondary antibody and visualized using West-Q Femto clean ECL solutions. For a loading control, the monoclonal anti-β-actin antibody (Genetex, GTX109639) at a dilution of 1:2,000 was used.

6. Evaluation of MNNG-induced mutations in organoids

After Organoids were exposed to MNNG, collected in cell recovery solution (Corning) and incubated at room temperature for 30 minutes with regular shaking in order to remove the Matrigel. The DNA from organoids was isolated using the DNeasy Blood & tissue kit (Qiagen) according to manufacturer's instructions. PCR was performed using AccuPower PCR Master Mix (Bioneer) according to manufacturer's protocol. Primer sequences: APC_CpG_1_for, 5'-AGACAAACAAGGATTTCCCGGAAGA-3', APC_CpG_1_rev, 5'-AGATGAACAATCATTTGCCAACAGA-3'; APC_CpG_2_for, 5'-TCATCACTCTGACAACTCAGTGACT-3', APC_CpG_2_rev, 5'-GCTCCTCGCCATGAATATGCTC-3'. The PCR products were Sanger sequenced by Macrogen.

7. Whole Genome Sequencing (WGS) and Read Alignment

The samples were prepared according to the Illumina TruSeq Nano DNA library preparation guide or TruSeq DNA PCR-free library preparation guide. The libraries were sequenced using Illumina platform. DNA libraries for sequencing from 1 μg of genomic DNA isolated from organoid by using the DNeasy Blood & tissue kit (Qiagen) according to manufacturer's instructions. First, paired-end sequences generated by the HiSeq instrument are mapped to the human genome using Isaac aligner (iSAAC-04.18.11.09) where the reference sequence is the UCSC assembly hg19 (original GRCh37 from NCBI, Feb. 2009).

III. RESULTS

1. Mismatch Repair (MMR) pathway

The MMR pathway primarily involves three steps: recognition, excision and resynthesis. In human cells, mismatch recognition is mediated predominantly by the heterodimer of hMSH2 and hMSH6, referred to as hMutSα.[8] Initially, the MutS protein complex recognizes base–base mismatches and insertion–deletion loops. This mismatch-bound hMSH2/hMSH6 heterodimer undergoes an ATP-dependent conformational change, which converts it to a sliding clamp capable of translocating along the DNA backbone. The hMSH2/hMSH6 ATP DNA complex is bound by a second heterodimer MutL, composed of hMLH1 and hPMS2.[9] This complex can translocate in either direction, in search of a strand discontinuity. The downstream consequences of these events include the induction of DNA damage signaling events, G2/M cell cycle arrest, induction of sister chromatid exchanges and apoptosis.[10] If the DNA lesion is not repaired, DNA replication by either conventional or translesion DNA synthesis (TLS) polymerases can lead to the formation of O^6meG-T mismatch. Recognition, following replication, of O^6-meG:C or O^6-meG:T mismatches by hMutSa and recruitment of hMutLa initiates futile cycles of excision repair. Replication past a gap left by removal of a mismatched T produces double strand breaks leading to cell death.
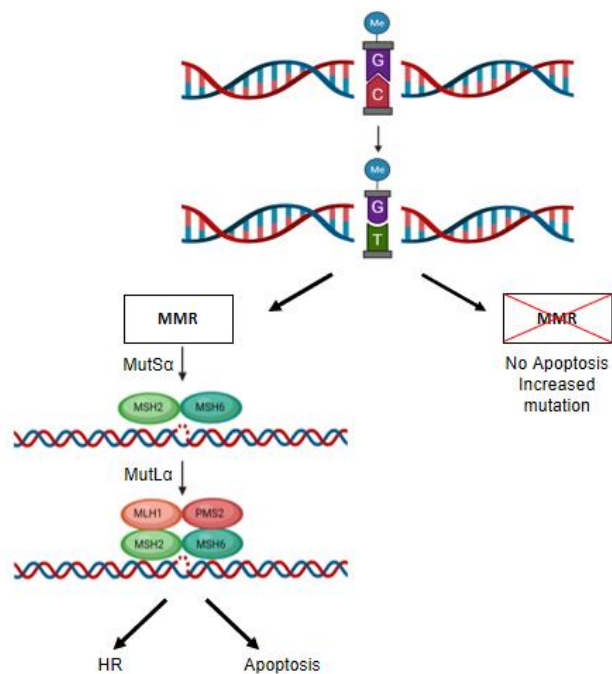
Figure 1. O⁶meG-T mismatch repair pathway. In MMR-deficient pathway, DNA mismatch escapes checkpoint signaling and accumulates G:C>A:T mutations at O⁶-meG:T sites during the following rounds of replication. The pathway results in cell survival but with mutation.[11]

2. Generation of the DNA damage lesion using methylating agent, MNNG

The pattern of DNA lesions generated by an alkylating agent depends on the number of reactive sites within the alkylating agent, its particular chemical reactivity, the type of alkyl group addition and the DNA substrate.[12] The O^6-position of guanine is a major site of methylation by S_N1 type alkylating agents for generating O^6-methylguanine (O^6-meG).[13] The O^6-meG pairs with thymine and induce the mismatch. Therefore, using SN1 type alkylating agent N-Methyl-N'-Nitro-N-Nitrosoguanidine (MNNG),[14] induces the O^6-meG lesion to generate DNA mispairs, then repairs the DNA lesion through the MMR pathway.[11]
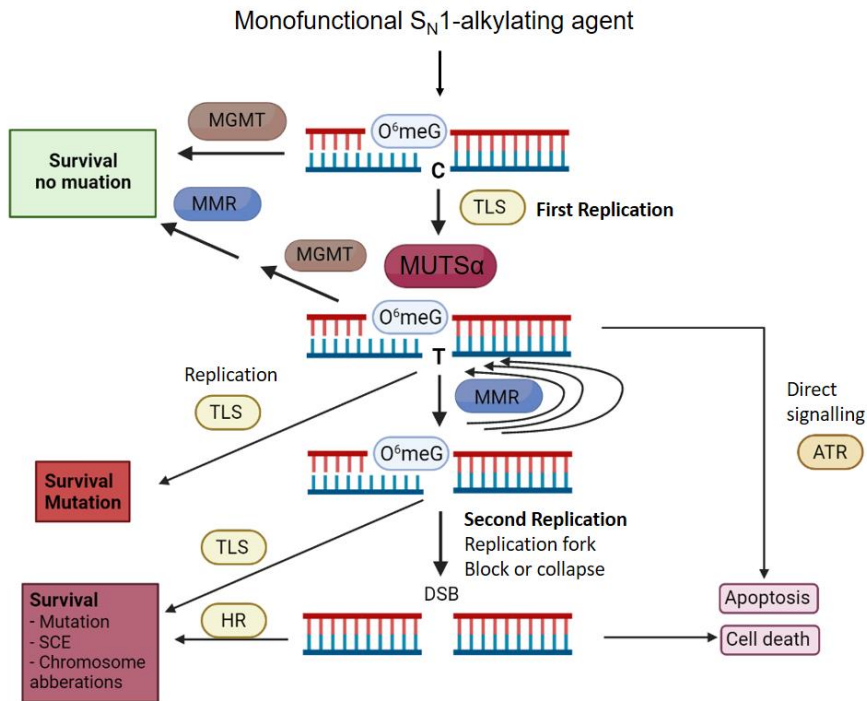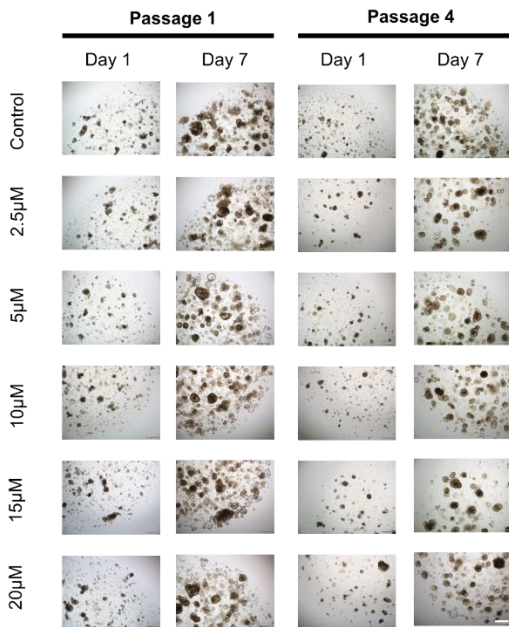
Figure 2. Repair of O⁶meG lesions created upon exposure to S_N1 DNA alkylating agents. S_n1-alkylating agents generate the O⁶-methylguanine and formation of O⁶meG:T mispairs. MUTSα recognizes the mispairs and initiates the MMR pathway, leading to futile cycles of DNA resection. The process can cause replication fort collapse and double-strand break (DSB). The DSB can be repaired by homologous recombination (HR) pathway resulting in cell survival but with mutation, sister chromatid exchange (SCE) or chromosome abberations.[13] If the DSB is not repaired, it can induce cell death by apoptosis.
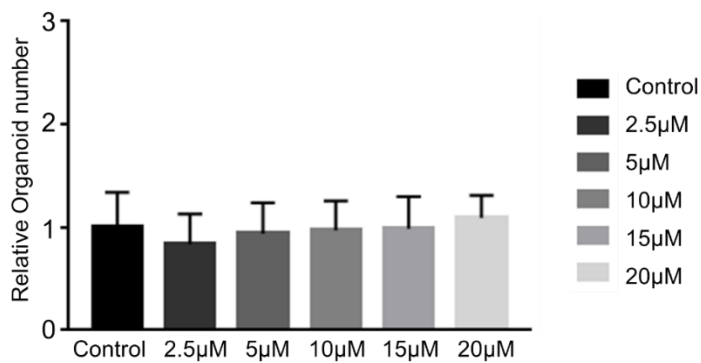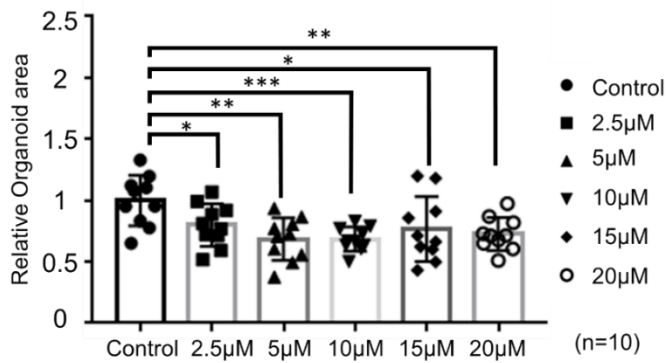
3. The effect of MNNG on growth of human normal colon organoids

The MMR process causes death of cells with methylation-damaged DNA bases. Therefore, to determine the proper dose and treatment time of MNNG for normal response as a control, we measured proportions of cell growth and death after treatment of the methylating agent, MNNG, in normal colon organoids. DNA lesion is caused by MNNG, which induces cell death through DNA damage. First of all, we treated high concentration (0, 2.5, 5, 15, 25 µM) of MNNG for short term (45 minutes) and low concentration (0, 0.5, 1, 1.5, 2.5 µM) for long term (48 hours). [6] The normal organoids were derived from normal colon tissue of colorectal cancer patients with MSS molecular feature. We observed the organoid growth at every passage after the treatment of MNNG, and compared the area and number of organoids after four passage of organoids (figure3 A-D). The organoid growth relatively decreased in MNNG-treated normal organoids but there was no significant difference in organoid number. (figure3 A-D). In addition, there was no significant difference in organoid growth between high dose/short term treated and low dose/long term treated organoids.
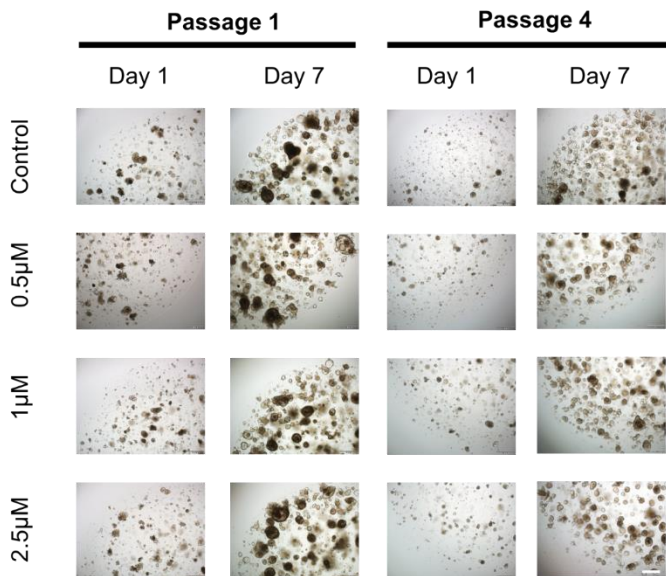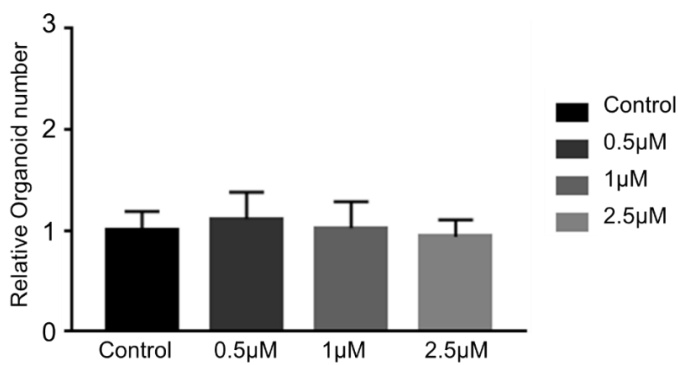
A.

B.

C.

D.

Figure 3. The effect of MNNG on growth of human normal colon organoids. (A, B) The MNNG was treated in concentration of 0, 2.5, 5, 15, 25 µM for 45 minutes. (A) Representative bright-field images of organoids. Scale bar, 500µm. (B) The organoids area and number were measured after 4 passages of organoids. (C, D) The MNNG was treated in concentration of 0, 0.5, 1, 1.5, 2.5 µM for 48 hours. (C) Representative bright-field images of organoids. Scale bar, 500µm. (D) The organoids area and number were measured after 4 passages of organoids. Data were expressed as the mean ± standard error of three independent experiments; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (compared with the control).

4. Repair of the DNA damage lesion by O-6-methylguanine-DNA methyltransferase (MGMT) and induction of apoptosis by ATR inactivation

The O^6-meG lesion is repaired by MGMT, and if MGMT is sufficient for repairing the whole O^6-meG lesions, the DNA mispairs are not made, and cells survive with no mutation.[15] Therefore, additional usage of O^6-Benzylguanine (O^6BG), the inhibitor of MGMT, can increase the sensitivity of MNNG and lead it to become more dependent on the MMR pathway. After MMR system processes, the ATR-chk1 activation participates in damage reparing, then without ATR-Chk1 activation, the apoptosis is induced. Therefore, the treatment of ATR inhibitor can lead to cell death.[16]

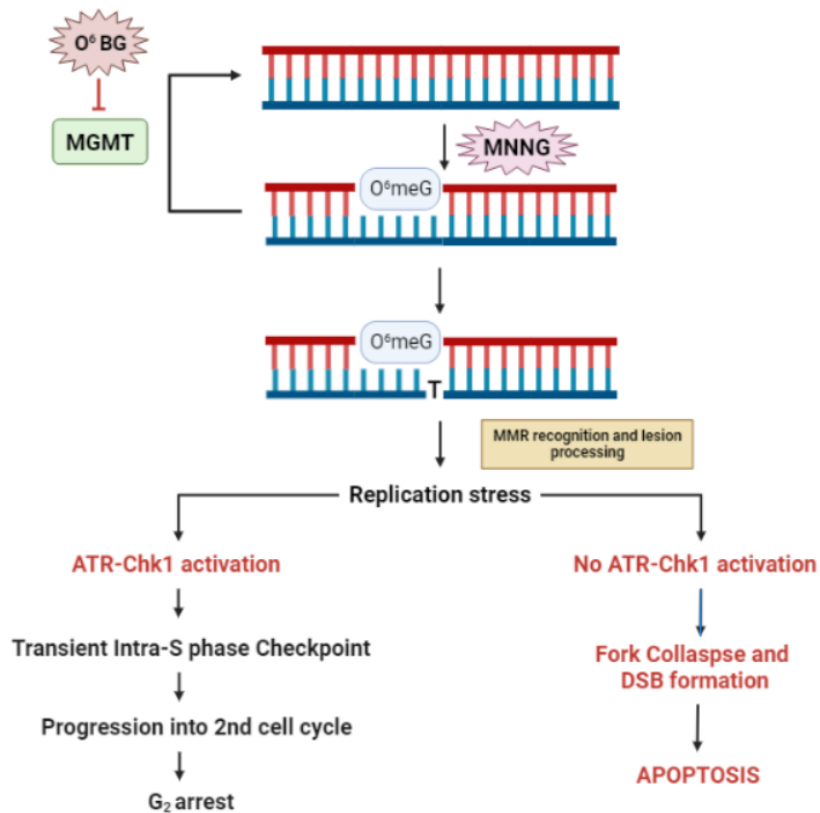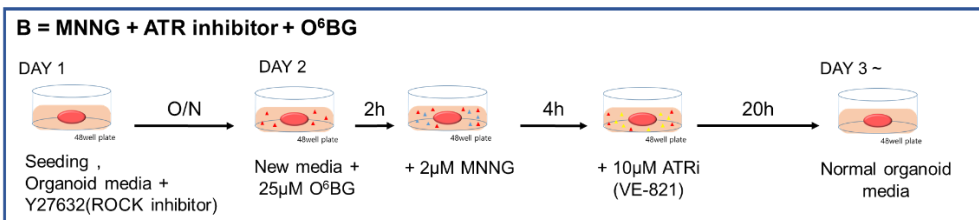**Figure 4. Increased MNNG-induced cell death by inhibition of MGMT and ATR.** The O^6-meG lesion can be directly repaired by O^6-methylguanine-DNA methyltransferase (MGMT). Therefore, O^6BG, the MGMT inhibitor, can increase MMR recognition and DNA lesion processing. Then, the MMR-dependent recognition of O^6-meG:T can directly signal for cell death through the inhibition of ATR interacting pathway.

5. The growth inhibition of MNNG-treated normal organoids by additional treatment of ATR inhibitor and O^{6}BG

To increase the sensitivity of MNNG and induce apoptosis in normal colon organoids, we additionally treated the O^{6}BG and ATR inhibitor (figure 4). We found that both the growth and number of organoids significantly decreased after the combined treatment of MNNG, ATR inhibitor and O^{6}BG in normal colon organoids (figure 5 B-C).

A.

A = MNNG + ATR inhibitor

DAY 1   DAY 2   DAY 3 ~

O/N   4h   20h

Seeding ,
Organoid media +
Y27632(ROCK inhibitor)

New media +
2µM MNNG

+ 10µM ATRi
(VE-821)

Normal organoid
media

B = MNNG + ATR inhibitor + O^{6}BG

DAY 1   DAY 2   DAY 3 ~

O/N   2h   4h   20h

Seeding ,
Organoid media +
Y27632(ROCK inhibitor)

New media +
25µM O^{6}BG

+ 2µM MNNG

+ 10µM ATRi
(VE-821)

Normal organoid
media

B.

C.

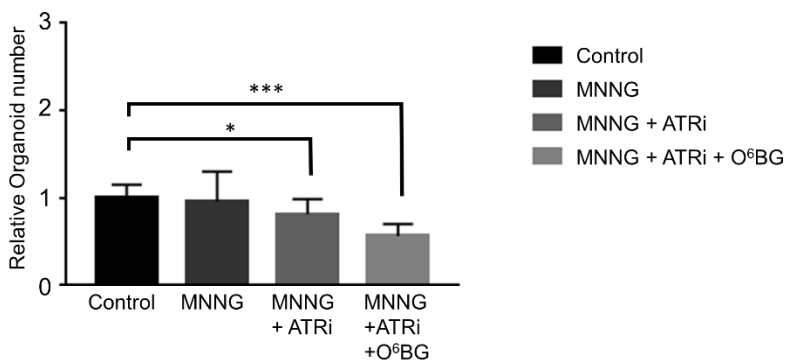**Figure 5. Growth suppression of normal colon organoids by combined treatment of MNNG, ATR inhibitor, and O⁶BG.** (A) A schematic diagram of drug treatment. With the pretreatment of O^{6}BG (25 µM) for 2 hours, the MNNG was treated in concentration of 2µM for 24 hours, and 10 µM of ATR inhibitor was treated. (B) Representative bright-field images of organoids after drug treatment. Scale bar, 500µm. (C) The organoids area and number were measured after 4 passages of organoids. Data were expressed as the mean ± standard error of three independent experiments; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (compared with the control).

6. No change of organoid growth by the combined treatment of MNNG, ATR inhibitor and O⁶BG in Lynch syndrome patient-derived organoids

Next, we treated the MNNG, ATR inhibitor and O^6BG in organoids derived from Lynch syndrome patients to confirm whether the MMR recognition of DNA lesion are working or not. (figure 6). Notably, there was no significant difference in organoid growth in both MLH1 and MSH2 mutated PDOs. These results suggest that the MMR-deficient organoids could escape the cytotoxic effect of combined agents because their MMR function was deficient.

A.

B.

C.

D.

Figure 6. No effect of combined treatment of MNNG, ATR inhibitor and O⁶BG on growth of the MMR gene mutated PDOs. With the pretreatment of O⁶BG (25 µM), the MNNG was treated in concentration of 2µM for 24 hours, then 10 µM of ATR inhibitor was treated. MLH1 gene mutated LS-PDOs (A, B) and MSH2 gene mutated LS-PDOs (C, D) were treated with combined drugs. (A, C) Representative bright-field images after drug treatment. Scale bar, 500µm. (B, D) The organoids area and number were measured after 4 passages of organoids. Data were expressed as the mean ± standard error of three independent experiments; * p < 0.05, ** p < 0.01, and *** p < 0.001 (compared with the control).
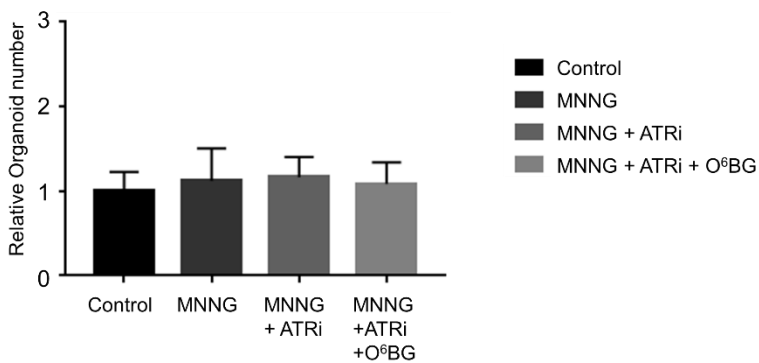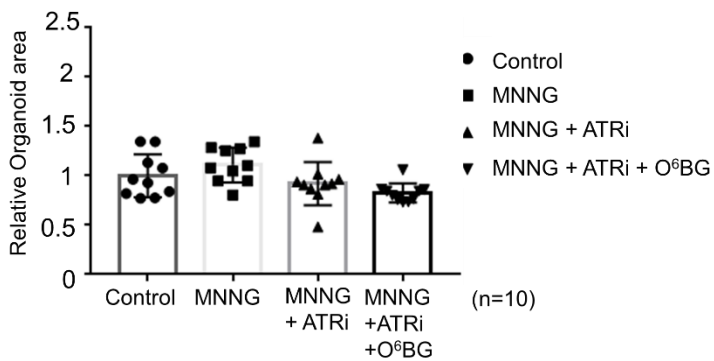
7. γH2AX expression as a key marker of DNA damage response

Although we found a significant difference in the growth of PDOs through the combined treatment of MNNG, O⁶BG and ATR inhibitor, we could measure this difference after several passage of organoids. Therefore, we need to search a molecular marker, which can be detected in earlier phase of DNA damage. H2AX is a key marker in the repair process of damaged DNA.[17] When DNA damage occurs, it is always followed by the phosphorylation of the histone H2AX, forming γH2AX (figure 7). Detection of γH2AX has emerged as a highly specific and sensitive molecular marker for monitoring DNA damage initiation and resolution. Quantitation of γH2AX foci has been applied as a useful tool for the evaluation of DNA damages by various drugs. Therefore, measuring γH2AX of MNNG-treated LS patient-derived organoids could be used for the evaluation of DNA damage by MMR deficiency, and its correlation with tumor risks in LS patients.

Figure 7. γH2AX, key molecular marker of DNA damage response. DNA damage is recognized by γH2AX as it initiates the DNA repair system. H2AX is phosphorylated by the protein kinase ATM which is activated in response to DSB. H2AX can also be phosphorylated by ATR. ATR phosphorylates H2AX in response to single-stranded DNA breaks and during replication stress. The ATM and ATR are central to the DNA damage response. Downstream of these proteins are two families of checkpoint kinases (CHEK), the CHEK1 and CHEK2 kinases. Below this level of signal transduction are the effectors that execute the functions of the DNA damage response.[18]

8. DNA Damage Responses (DDR) of PDOs to MNNG and O⁶BG

To evaluate the expression of γH2AX in normal, MLH1 mutated and MSH2 mutated patient-derived organoids, we treated MNNG (25µM) or combination of MNNG (25µM) and O⁶BG (25µM). Then, we confirmed the expression of γH2AX by immunohistochemistry (figure 8A), western blot (figure 8B) and immunofluorescence (figure 8C). We performed immunofluorescence in normal organoids from 6 patients, MLH1 mutated organoids from 5 LS-patients and MSH2 mutated organoids from 5 LS-patients (figure 8 D-F). Thus, we found the increased γH2AX expression after treatment of MNNG or combination of MNNG and O⁶BG in normal organoids. However, the PDOs with the MMR gene mutation showed no or less DNA damage responses after the drug treatment, compared to normal organoids.

A.

2 µM MNNG

γH2AX

Normal organoid

MLH1 mutation Organoid

MSH2 mutation Organoid

B.

C.

D.

	MNNG 10µM	MNNG 10µM + O^{6}BG	MNNG 25µM	MNNG 25µM +O^{6}BG
Patient A	n.s (0.5647)	* (0.0077)	* (0.018)	*** (0.0009)
Patient B	n.s (0.2235)	* (0.0160)	* (0.0218)	*** (0.0003)
Patient C	n.s (0.1784)	n.s (0.1332)	** (0.0016)	n.s (0.1017)
Patient D	n.s (0.0607)	n.s (0.1199)	* (0.0309)	n.s (0.2175)
Patient E	n.s (0.8714)	n.s (0.2136)	n.s (0.0595)	* (0.0064)
Patient F	n.s (0.2514)	n.s (0.0608)	* (0.0225)	* (0.0138)

*p<0.05, **p<0.01, **p<0.001, compared with the control

E.

	MNNG 10μM	MNNG 10μM + O^{6}BG	MNNG 25μM	MNNG 25μM +O^{6}BG
Patient G	n.s (0.1003)	n.s (0.1802)	n.s (0.2053)	n.s (0.3056)
Patient H	* (0.0260)	n.s (0.1604)	n.s (0.1176)	n.s (0.1084)
Patient I	n.s (0.1662)	n.s (0.6167)	n.s (0.4530)	n.s (0.0894)
Patient J	n.s (0.3331)	n.s (0.6012)	* (0.0157)	n.s (0.3827)
Patient K	n.s (0.4711)	n.s (0.0598)	n.s (0.1426)	** (0.0017)

*p<0.05, **p<0.01, **p<0.001, compared with the control

F.

	MNNG 10μM	MNNG 10μM + O^{6}BG	MNNG 25μM	MNNG 25μM +O^{6}BG
Patient L	* (0.0316)	n.s (0.8441)	n.s (0.9522)	* (0.0436)
Patient M	n.s (0.1516)	n.s (0.9853)	n.s (0.3320)	n.s (0.4917)
Patient N	n.s (0.1202)	n.s (0.7620)	n.s (0.6542)	n.s (0.5011)
Patient O	n.s (0.7492)	n.s (0.3699)	* (0.0304)	* (0.0327)
Patient P	* (0.0261)	* (0.0049)	n.s (0.3926)	n.s (0.0711)

*p<0.05, **p<0.01, **p<0.001, compared with the control

Figure 8. ɣH2AX expression after treatment of MNNG and O⁶BG in normal and LS-PDOs. (A) Immunohistochemical staining, (B) western blot analysis and (C) immunofluorescence staining for γH2AX expression were performed in normal organoids and MLH1/MSH2 mutated LS-PDOs after treatment of 2µM MNNG or 25 µM MNNG and O⁶BG for 24 hours. Scale bar, 50µm (A) and 20 µm (C). (D-F) The expression of γH2AX after MNNG and O⁶BG treatment were measured by immunoflouorescence in normal organoid (D), MLH1 mutated organoid (E) and MSH2 mutated organoid (F) LS-PDOs. (top, the immunoflouorescene images of organoids after drug treatment; bottom, relative γH2AX/DAPI intensity curves after drug treatment) Scale bar, 100 µm. Data were expressed as the mean ± standard error of three independent experiments; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (compared with the control).

9. MNNG-induced mutation accumulation in normal organoids and LS-PDOs

MNNG induces O^6-meG and it pairs with thymine mismatch, and the MNNG-treated cells initially show C>T base transitions. Therefore, we performed Sanger sequence analysis to identify the mutations accumulated by MNNG. To clearly identify the base transitions, we targeted sequences in 2 sites of CpG island of APC gene. The organoids were treated with 25μM MNNG for 24 hours and harvested after 0 hour, 24 hours and 48 hours. As expected, after the MNNG treatment, MLH1 and MSH2 mutated LS-PDOs showed an increased C>T base transitions over time, compared to normal organoids.

A.

25μM MNNG treatment

| 0H | 24H | 48H | 72H |

Control — Normal culture condition → **Organoid Harvest**

0H — Normal culture condition / MNNG treatment

24H — Normal culture condition / MNNG treatment / Recovery

48H — MNNG treatment / Recovery

C>T

TCCNNCATTNNC

B.

C.

D.

Figure 9. MNNG-induced Single Nucleotide Variants(SNVs) in normal organoids and LS-PDOs. (A) A shematic diagram of drug treatment and organoid harvest with recovery time (top), Sanger sequencing analysis of C>T transitions (bottom). The accmulated number of C>T transition of normal (B), MLH1 mutated (C) and MSH2 mutated (D) organoids with 25μM MNNG treatment.

Table1. Primer sequences for CpG island in APC gene.

Target	Primer Sequence(5' to 3')	GC%	Length
APC_CpG_1	Forward: AGACAAACAAGGATTTCCCGGAAGA	57	1430
	Reverse: AGATGAACAATCATTTGCCAACAGA		
APC_CpG_2	Forward: TCATCACTCTGACAACTCAGTGACT	54	923
	Reverse: GCTCCTCGCCATGAATATGCTC		

10. Whole genome sequence analysis in MNNG-treated normal organoids and MLH1 mutated PDOs

To idenitfy the mutations that accumulated by MNNG, the normal organoid and MLH1 mutated LS-PDO were subjected to whole genome sequencing(WGS) analyse after treatment of MNNG. We treated 25µM of MNNG in normal and MLH1 mutated PDOs for 24 hours and cultured without MNNG for 48 hours to allow organoids to accumulate or recover muations. The mutation is accumulated in MLH1-deficient organoids, which is driven by replication errors.[19] Both MNNG-treated and untreated organoids were subjected to WGS analyses to identify the muatations that accumulated in the normal and MLH1 mutated PDOs. The MNNG-treated normal organoids showed an decreased number of base subsitution compared to MNNG-untreated oragnoids. However, the MLH1 mutated organoids showed an increased number of base subsitution in MNNG treatment, compared to MNNG-untreated organoids. This confirmed that pathogenic mutation of MLH1 gene induced the generation of muations by dysfunction of mismatch repair.

A.

Control

MNNG treatment

B.

Control

MNNG treatment

Figure 10. MNNG-induced Mutation analysis in normal and MLH1 mutated PDOs. Number of mutations accoumulated in normal oraganoids (A) and MLH1 mutated oraganoids (B) in both MNNG-treated and untreated condition was measured by WGS. Shown are variants subdvidived by mutation type, SNP, INDELs and base substitutions. (top, the analysis of mutations MNNG-untreatment; bottom, the analysis of mutations after 25 μM MNNG treatment for 24 hours and recovery for 48 hours)

IV. DISCUSSION

Although LS patients have a high risk of CRC, they have various risk of developing CRC.[20] The LS is caused by inherited mutations in genes of the DNA mismatch repair (MMR) pathway, which has been remarkably important for the management of this disease. However, the proper risk assessment that evaluates the cancer risk of LS patients has not been established. Also, a proper diagnosis, prevention approaches, and appropriate decisions for LS including aggressive CRC patients are needed. Therefore, detailed molecular information about the MMR pathway has been essential for guiding the diagnosis of LS. Recently, it has been reported that the MMR-deficient cells are more resistant to the cytotoxic effects of certain DNA damage agents. In particular, Karran P. et al demonstrated that MMR-deficient *E.coli* were shown to be resistant to the cytotoxic effects of the DNA alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG).[21] MNNG has been used to induce DNA methylation damage that is directly recognized by MMR proteins, which results in recruitment of MMR genes. Thus, the critical cytotoxic lesion created by MNNG is the O^6-methylguanine, which is commonly mispaired with thymine during replication resulting in a MeG-T mispair. Then MMR-related genes recognize and remove these lesions, and methylguanine methyltransferase (MGMT) also directly repairs methylguanine. Thus, the processing of a MeG-T mispairs by the MMR pathway creates secondary DNA damage that ultimately causes cell death. In addition to the futile cycle model, researchers have demonstrated protein-protein interactions between the MMR proteins and key DNA damage signaling molecules such as ATR, ATM, CHK1, and CHK2. These mechanisms indicate that MMR plays a protective role in repairing and removing damaged cells to reduce the risk of mutation accumulation.

Using these detailed molecular mechanisms of MMR pathway, we came to identify some factors to be considered for developing an individualized model to predict CRC risk in LS.

As for the biological significance of MMR genes, some researchers showed functional assays in different experimental systems. However, most of them have

intrinsic limitations because they were developed based on bacteria, yeast, or mice.[7]

Recently, Bouvet D et al. reported that an assay for measuring cell response to the cytotoxic effect of a methylating agent can determine the effects of VUS in MMR genes. Based on the DNA damage-induced apoptosis function of MMR, they evaluated the significance of VOUS of MMR genes, using a large panel of 88 variants, mainly missense variants, including a validation set of 40 previously classified variants and a prospective set of 48 VUS.[6] However, this assay also measured only restrictive cell biological properties of variant MMR protein levels.

Therefore, we used LS-PDOs to reflect their own other molecules to be related to the MMR pathway and its consequence. Then, to maximize the difference in MNNG-induced cytotoxicity of organoids, we used an ATR inhibitor. In addition, to investigate the functional activity of MGMT to repair methylguanine and suppress their function to show more MMR-dependent pathways, O^6BG, an MGMT inhibitor, was used in our models. Therefore, we utilized normal organoids and LS-PDOs treated with ATR inhibitor and O^6BG to maximize the difference in MNNG-induced cytotoxicity. Then, in the PDO model for earlier detection of DDR using γH2AX, organoids were treated with MNNG alone and MNNG/O^6BG, and without ATR inhibition to detect DDR in live cells.

From our results, we demonstrated that MMR gene mutated LS-PDOs could survive in MNNG-induced DNA lesions and segmented cell death, which suggest the association with high cancer risk.

However, to confirm the difference of MNNG-induced cytotoxicity in PDOs, long-term culture of organoids for several passages was needed. Therefore, to detect DNA damage in an earlier phase, we utilized a DNA damage recognition marker, γH2AX. Normal organoids showed high DDR by MNNG and O^6BG treatment. However, the MMR gene mutated organoids showed a much less DDR. Our results enabled the discrimination of early phase DDR damaged by MNNG and O^6BG between normal and LS patients. Then, we confirmed the difference in mutation accumulation

between normal and LS-PDOs by Sanger sequencing analysis of CpG island site, and WGS in the same condition of drug treatment. In addition, our analysis of sequencing data allowed us to define a mutational signature of MMR deficiency.

However, even in normal organoids, there were various MNNG-induced DDR changes. Therefore, we need to explain these variations by IHCS of MMR proteins to confirm the correlation between DDR and MMR protein expression levels in normal organoids.[22]

In addition, recently, Lena Bohaumilitzky, et al., (2022) demonstrated an increased T-cell infiltrate in the normal colon mucosa of MSI CRC patients and healthy LS carriers compared with MSS CRC patients.[23] LS carriers showed elevated mucosal T-cell infiltration even without cancer. These results suggest that we have to consider the factor of immune rejection as a protective factor against CRC risks in LS patients in future studies.

Further research using larger sample sizes with long-term prognostic clinical data and various genetic mutations associated with LS is needed to confirm that our PDO model is suitable for CRC risk prediction in LS patients. In addition, Jarno Drost, et al have shown that the pre-dominant mutation profiles observed in MLH1 deleted organoids by using CRISPR/Cas9 technology.[19] This technology also could be useful to investigate the biological and clinical significance of MMR genes.

Moreover, our PDO model can be applied to biological validation of VOUS, and individualized cancer risk prediction models of other tumors, such as endometrial and gastric cancer as well.

V. CONCLUSION

In conclusion, we demonstrated that MNNG-induced DNA damage and cell death were different between normal organoid and LS-PDOs, suggesting that our PDO model could serve as a useful individualized prediction model for CRC risk in LS patients.

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ABSTRACT(IN KOREAN)

불일치 복구 유전자의 생식세포 돌연변이 환자에서 환자 유래 오가노이드 모델을 이용한 대장암 위험도 예측

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신유미

대장암(CRC)의 약 10~15%가 현미부수체 불안정성(MSI) 종양을 보이고, MSI 종양의 약 20%가 '린치증후군 (Lynch syndrome)' 또는 '유전성 비용종증 결장직장암 증후군 (HNPCC)'으로 알려진 불일치 복구 (MMR) 유전자 MLH1, MSH2, MSH6, PMS2, EPCAM의 생식선 돌연변이에 의해 발생된다.

린치증후군 환자는 대장암 발병 위험이 높지만, 린치증후군 환자에서 암위험도는 실제 다양하게 나타나며, 암위험도를 예측할 수 있는 효과적인 모델이 없다. 따라서, 우리는 린치 증후군 환자 유래 오가노이드 (PDO)를 이용한 대장암 발병 위험도에 대한 개별화된 예측 모델을 계발하고자 한다.

우선 DNA 손상 유발 세포사멸반응을 기반으로 메틸화제인 N-메틸-N'-니트로-N-니트로소구아니딘 (MNNG)의 세포독성 효과에 대한 오가노이드 반응을 측정했다. 정상 대장 오가노이드에서 고농도/단기간 및 저농도/장기간의 MNNG를 처리하였고, 오가노이드 크기는 상대적으로 감소했지만 갯수에는 큰 차이를 보이지 않았다. MNNG의 효과를 높이고 대장 오가노이드의 세포 사멸을 유도하기 위해 O^6BG와 ATR 억제제를 추가로 처리하였다. MNNG, O^6BG 및 ATR 억제제를 같이 처리함으로써 세포독성 효과에서 MMR 유전자 돌연변이 오가노이드에 비해 정상 오가노이드가 민감하다는 것을 발견했다. 더 이른 단계에서의 빠른 변화의 차이 확인을 위해 DNA 손상 인식 마커인 γH2AX의 발현을 분석하여 DNA 손상 반응을 조사하였다. MGMT 억제제인 MNNG와

O^{6}BG를 처리한 후, MMR 유전자 돌연변이 오가노이드보다 정상 오가노이드에서 γH2AX의 높은 발현을 발견했다. 또한, MNNG에 의해 유도된 돌연변이 수가 정상 오가노이드에 비해 MMR 유전자 돌연변이 오가노이드에서 상당히 증가하는 것을 발견했다.

MNNG에 의해 축적된 돌연변이를 확인하기 위해, 생어시퀀싱 분석과 전장유전체분석 (Whole Genome Sequencing)을 진행하였다. 대장 오가노이드에 MNNG를 처리하고, 여러 회복 시간 별로 오가노이드를 얻어 분석을 진행하였다. 정상 오가노이드에서 시간에 따라 감소되는 염기 전위들을 발견할 수 있었지만 반면에, MMR 유전자 돌연변이 오가노이드에서는 증가되는 것을 발견했다. 더불어, 전장유전체분석 결과를 통해, MNNG에 의해 유도된 돌연변이 숫자가 MLH1 유전자 돌연변이 오가노이드에서 정상 오가노이드에 비해 상당히 증가되는 것을 발견하였다.

결론적으로, 린치 증후군 환자 유래 오가노이드 모델에서 MNNG와 O^{6}BG에 의한 DNA 손상 반응 측정은 대장암 위험의 개별화된 예측 모델이 될 수 있다.

핵심되는 말 : 환자 유래 오가노이드, 대장암, 린치신드롬, **DNA** 손상 반응, **MNNG**