



# Changing of gamma-H2AX in peripheral blood mononuclear cells during concurrent chemoradiation in locally advanced rectal cancer patients: a potential response predictor

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**Background:** The most detrimental effect of DNA damage from radiation is DNA double-strand breaks, making it critical to identify reliable biomarkers for treatment response in cancer therapy. Gamma-H2AX ( $\gamma$ -H2AX), a marker of DNA double-strand breaks, was evaluated in this study as a potential biomarker for treatment response in locally advanced rectal cancer patients undergoing preoperative concurrent chemoradiation (CCRT).

**Methods:** Thirty patients with locally advanced rectal cancer received preoperative CCRT. Peripheral blood mononuclear cells (PBMCs) were collected at five time points: baseline, 24 hours after the first radiation fraction, mid-treatment, end of treatment, and six weeks post-CCRT.  $\gamma$ -H2AX levels were measured in these samples. MRI was used to assess treatment response based on magnetic resonance tumor regression grade (mrTRG). Patients were classified as responders or non-responders based on mrTRG. *T*-test and repeated measures analysis of variance (ANOVA) evaluated dynamic changes in  $\gamma$ -H2AX levels, and a multilevel linear regression model analyzed the relationship between  $\gamma$ -H2AX levels and treatment response.

**Results:** Nineteen out of thirty patients (63.33%) were classified as responders. Significant dynamic changes in  $\gamma$ -H2AX levels were observed between non-responders and responders ( $P=0.01$ ). The multilevel linear regression model showed a trend towards increased  $\gamma$ -H2AX levels in responders [1.17, 95% confidence interval (CI): -0.02 to 2.34,  $P=0.053$ ]. Significant differences in  $\gamma$ -H2AX levels were observed from baseline to mid-treatment, end of treatment, and six weeks post-CCRT. Pathologic complete response (pCR) after CCRT was associated with significantly higher  $\gamma$ -H2AX ratios compared to those without pCR ( $P=0.04$ ). However, no significant difference was identified in the multilevel linear regression model.

**Conclusions:**  $\gamma$ -H2AX may have potential as a biomarker for treatment response in locally advanced rectal cancer patients undergoing preoperative CCRT, although further validation is required.

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**Keywords:** Gamma-H2AX ( $\gamma$ -H2AX); preoperative concurrent chemoradiation; rectal cancer; DNA double strand break; tumor regression grade (TRG)

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## Introduction

Rectal cancer is the eighth most commonly diagnosed cancer and the tenth leading cause of cancer-related deaths worldwide (1). The standard therapeutic approaches for locally advanced diseases is total neoadjuvant therapy (TNT) which includes preoperative concurrent chemoradiation (CCRT) and chemotherapy followed by total mesorectal excision (TME). The addition of chemotherapy following CCRT, had demonstrated significant improvements in the pathologic complete response (pCR) of patients during surgery (2,3). However, some patients may not tolerate consolidative chemotherapy as planned. Multidisciplinary treatments are now considered essential for the management of rectal cancer (4).

High resolution magnetic resonance imaging (MRI) of the rectum is a standard imaging option for preoperative rectal cancer assessment and can provide superior and accurate information on the primary tumor stage and mesorectal fascia involvement (5,6). Furthermore, post-

CCRT MRI has been increasingly used for radiological response evaluation to assess tumor volume reduction and any signals of fibrosis. With regard to the MERCURY study, the clinical tumor response has been classified by the magnetic resonance tumor regression grade (mrTRG) system. Patients who achieved a good response exhibited significant outcomes in terms of being disease-free and improved chances for overall survival when compared with those who received a poor response (7). In terms of both clinical staging and the response after the administration of preoperative measures, CCRT could be a key step in developing more individualized treatment for each patient (8). Apart from MRI assessment, several biomarkers have increasingly been utilized for the prediction of treatment response for radiotherapy. Although many kinds of blood sample biomarkers have been tested in clinical trials, any prediction or correlation of the response to neoadjuvant CCRT have been controversial (9,10).

To define the radiosensitivity of a tumor, several biomarkers have been used in previous studies. Gamma-H2AX ( $\gamma$ -H2AX), one of the early indicators of DNA double strand breaks (DSBs), is a phosphorylated form of the H2AX protein in DNA histone cores that occurs after radiation has been used as the primary determinant for tumor response to irradiation in both normal and cancerous cells (11-15). Studies investigating  $\gamma$ -H2AX as a surrogate marker of DNA double-strand damage have been inconclusive in predicting tumor response in several cancer types (13,16). Only one study has collected peripheral blood mononuclear cells (PBMCs) from rectal cancer patients treated with radiotherapy, but it included only one PBMCs collection after five fractions of radiotherapy (11). Given the limitations of current biomarkers in predicting response to preoperative CCRT, there is a critical need for identifying novel biomarkers that can accurately assess treatment response. In this context, our study aims to investigate the dynamic change of  $\gamma$ -H2AX in PBMCs as a potential biomarker for monitoring treatment response in patients diagnosed with locally advanced rectal cancer undergoing preoperative CCRT. The expression percentage of  $\gamma$ -H2AX

### Highlight box

#### Key findings

- Gamma-H2AX ( $\gamma$ -H2AX) levels in peripheral blood mononuclear cells (PBMCs) show significant dynamic changes between responders and non-responders to concurrent chemoradiation (CCRT) in locally advanced rectal cancer patients.

#### What is known and what is new?

- $\gamma$ -H2AX is a known marker for DNA double-strand breaks caused by radiation.
- This study adds evidence that  $\gamma$ -H2AX levels could be used as a biomarker for predicting treatment response in rectal cancer patients undergoing preoperative CCRT.

#### What is the implication, and what should change now?

- The findings suggest potential clinical use of  $\gamma$ -H2AX levels to tailor treatment strategies for rectal cancer patients.
- Further validation is required to confirm  $\gamma$ -H2AX as a reliable biomarker, which could lead to personalized and more effective cancer treatment protocols, improving patient outcomes and optimizing therapy efficiency.

in PBMCs during preoperative CCRT was measured using the flow cytometry technique. We present this article in accordance with the STROBE reporting checklist (available at <https://jgo.amegroups.com/article/view/10.21037/jgo-24-488/rc>).

## Methods

### *Study population and participant recruitment*

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Faculty of Medicine, Chiang Mai University Ethics Committee for Human Research (protocol code RAD-2563-07543, September 25th, 2020). Locally advanced rectal cancer patients who underwent preoperative CCRT treatment at the Division of Radiation Oncology, Department of Radiology, Faculty of Medicine, Chiang Mai University between November 2020 and November 2021 were enrolled in this study. All patients had given verbal and written informed consent for their participation in this research study. Eligibility criteria included patients with newly diagnosed rectal adenocarcinoma confirmed by histopathology, who were classified as clinical stage T3 or T4, or had positive indications for regional lymph node involvement, and were candidates for preoperative CCRT. Patients were also included who exhibited normal liver function, displayed creatinine clearance for at least 30 mL/min, and had received an Eastern Cooperative Oncology Group (ECOG) performance status within the range of 0–2. Patients who had a history of previous pelvic irradiation, had recurrent rectal cancer, received any systemic cancer treatment, or who had exhibited uncontrolled medical conditions were totally excluded. All patients underwent complete laboratory and staging investigations including an MRI in the pelvic region and a computed tomography (CT) scan of the chest-abdomen-pelvis area.

### *Study protocol*

All enrolled patients received preoperative CCRT that involved intensity modulated radiotherapy (IMRT) technique using helical tomotherapy and chemotherapy. Target volume [gross tumor volume (GTV); clinical target volume (CTV); planning treatment volume (PTV)] and organs at risk (OARs) were contoured according to International Consensus Guidelines on clinical target

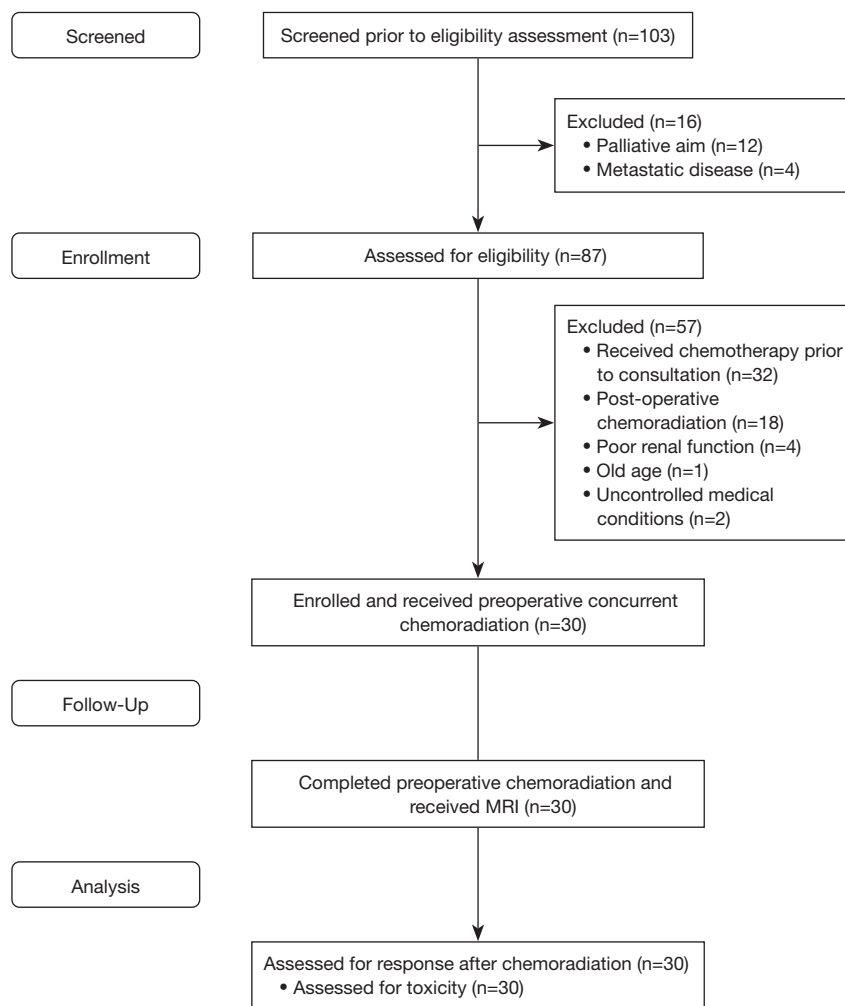
volume delineation for rectal cancer (17). The treatment regimen included simultaneous integrated boosts of radiation doses at 50 Gy to GTV (gross tumor and pathologic lymph node) and 45 Gy to regional lymph node CTV in 25 fractions. The chemotherapeutic agent that was given concurrently in this clinical study was capecitabine (825 mg/m<sup>2</sup> twice daily) and was administered on the day that the patient received radiotherapy (18). Patients who had creatinine clearance of lower than 50 mL/min received 75% of the standard dose of capecitabine. During CCRT, all patients were routinely appointed for a physical examination once a week in order to monitor and record any acute radiation side effects using Common Terminology Criteria for Adverse Events (CTCAE) version 5.0 (2017). The CONSORT flow-chart of the present study shown in *Figure 1*. After completing the study protocol, patients were referred to a colorectal surgeon for evaluation and consideration of oncologic surgery. Follow-up appointments were scheduled every three months.

### *γ-H2AX analysis*

Patient's blood samples of about 10 mL for serum γ-H2AX were collected 5 timepoints as showed in *Figure 2*. Immediately after blood collection, DNA damage evaluation based on γ-H2AX level in PBMCs through flow cytometry technique. Briefly, PBMCs were separated from the blood by centrifugation at 800 ×g for 30 minutes at 10 °C. After the separation, a layer of PBMCs was isolated and wash three times with phosphate-buffered saline and centrifuged for five minutes at 800 ×g. The isolated PBMCs were counted (approximately 2×10<sup>5</sup> cells) and diluted with assay buffer. Afterwards, the cells were fixed, permeabilized and incubated with anti-phosphohistone H2AX and anti-H2AX, and then analyzed the percentage of phosphorylated Histone H2AX by the Muse™ cell analyzer following the manufacturer's instructions. The report for the analyzer provided the percentage of γ-H2AX activation. Considering the variability in baseline γ-H2AX activation for each patient, we utilized the γ-H2AX activation ratio to compare levels at 2<sup>nd</sup>, 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> PBMCs collection to the baseline (1<sup>st</sup>) level.

### *Response assessment using MRI*

All patients underwent high-resolution pelvic MRI before CCRT and 4–6 weeks after receiving CCRT using 1.5-T and 3.0-T magnetic resonance machines. The initial MRI

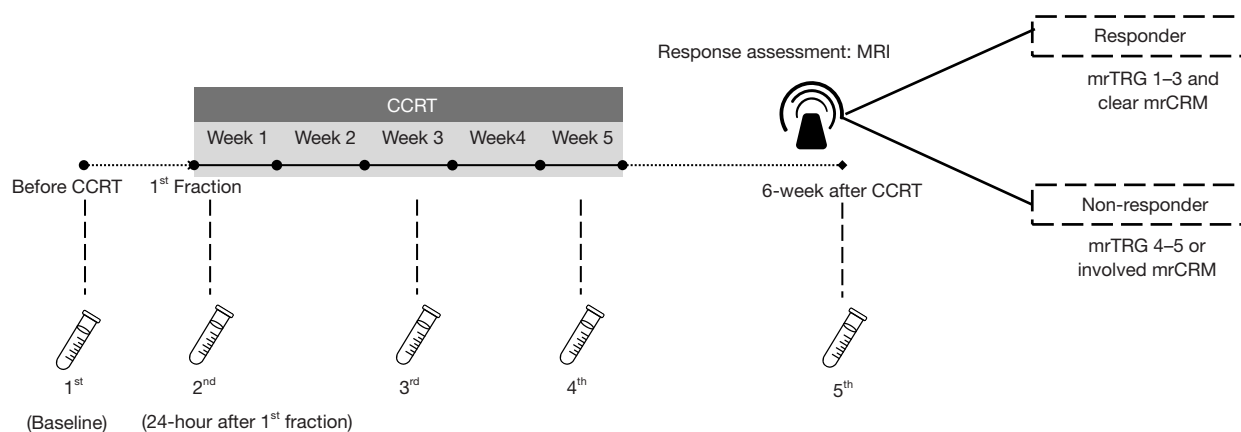


**Figure 1** The CONSORT flow-chart. MRI, magnetic resonance imaging.

protocol for tumor staging included large field of view (FOV) in sagittal and axial images and small FOV for high-resolution oblique axial image perpendicular to the long axis of tumor, sagittal and coronal on T2-weighted sequence, T1-weighted sequences, and diffusion weight image (DWI) sequences. Oblique coronal T2-weighted images were obtained for low rectal tumor. The same MRI protocol was obtained to assess tumor response after CCRT with additional pre- and dynamic post-gadolinium enhancement T1-weighted images.

The tumor (T) and nodal (N) staging, presence of extramural vascular invasion (EMVI) and the magnetic resonance circumferential resection margin (mrCRM) before and after CCRT were evaluated. For post CCRT MRI study, the extent and degree of post-treatment fibrosis was identified and the magnetic resonance tumor regression

grade (mrTRG), using MERCURY system were assessed and recorded by the same experienced gastrointestinal radiologist, who is a specialist with 20 years of experience in radiology. This radiologist has been utilizing the MERCURY system for mrTRG assessments since 2019, bringing 2 years of specific experience with this criterion. Post treatment fibrosis was identified as low signal on T2-weighted image. The residual tumor appeared as intermediate signal intensity on T2-weighted image similar to those of tumor on pre-CCRT image. The MRI response was used to divide patients into 2 groups as shown in *Figure 2*; the first group was comprised of good responders who were defined as mrTRG 1–3 and clear mrCRM, while the second group was comprised of poor responders who were defined as mrTRG 4–5 or who had involved mrCRM results (7).



**Figure 2** Flowchart of the study. PBMCs collection was collected before, during and after CCRT. MRI was performed to evaluate treatment response. Patients were divided into two groups based on their response. Responder included patients with mrTRG scores of 1–3 and clear mrCRM. Non-responder comprised patients with mrTRG scores of 4–5 or involved mrCRM. PBMCs, peripheral blood mononuclear cells; CCRT, concurrent chemoradiation; MRI, magnetic resonance imaging; mrTRG, magnetic resonance tumor regression grade; mrCRM, magnetic resonance circumferential resection margin.

### Statistical analysis

Statistical analyses were conducted using Stata version 16 for Windows. Fisher's exact test and Mann-Whitney *U* test were used to identify differences between imaging response and patient characteristics. In the univariable analysis of  $\gamma$ -H2AX activation, *t*-test and repeated measures analysis of variance (ANOVA) were employed to test differences between group of response. Considering the variability in measurement intervals and multiple confounding factors, a multilevel linear regression model was utilized, incorporating random intercept and fixed slope for each patient to account for individual variability, and marginal prediction plots were generated to visualize the predicted trend of  $\gamma$ -H2AX activation ratio changes from the 1<sup>st</sup> to 5<sup>th</sup> PBMCs collection. A *P* value less than 0.05 was considered statistically significant.

Additionally, Cox proportional hazards models were used to examine the association between  $\gamma$ -H2AX activation ratios and distant metastasis-free survival as well as overall survival. Correlations between  $\gamma$ -H2AX activation ratios and white blood cell (WBC) counts and lymphocyte counts were assessed using Pearson correlation.

## Results

### Patient characteristics

Thirty locally advanced rectal cancer patients were enrolled

in this study. All patients received preoperative CCRT without treatment interruption. Among the patients analyzed, 21 patients (70%) were male and 9 (30%) were female with a median age of 61 years (ranging from 23 to 79 years). Most patients had well-differentiated adenocarcinoma (56.67%). According to the MRI findings after CCRT using the MERCURY system, 63.33% (*n*=19) were classified as responders, while 36.67% (*n*=11) were classified as non-responders. Patient characteristics for each response group are summarized in *Table 1*.

### $\gamma$ -H2AX profile

The  $\gamma$ -H2AX activation for all patients was illustrated in *Figure 3*. A substantial increased  $\gamma$ -H2AX activation in PBMCs was noted throughout the treatment course, as determined by repeated measures ANOVA (*P*=0.007, *Figure 3A*). Additionally, significant differences were observed between response groups (*P*=0.01, *Figure 3B*). The crude mean activation ratio of  $\gamma$ -H2AX showed in *Table 2*.

The ratio of  $\gamma$ -H2AX activation assessed using a multilevel linear regression model that accounted for confounding factors, revealed higher expression trend in the responder group compared to non-responder 1.17 [95% confidence interval (CI): -0.02 to 2.34, *P*=0.053] (*Table 2*). The differences between baseline (1<sup>st</sup>) and the 2<sup>nd</sup> (0.60, 95% CI: -0.21 to 1.40, *P*=0.15), the 3<sup>rd</sup> (0.97, 95% CI:

**Table 1** Patient characteristics

Factors	Total (n=30)	Non-responder (n=11)	Responder (n=19)	P value
Age (years), median [IQR]	61 [54–67]	59 [48–71]	62 [56–67]	0.83 <sup>b</sup>
Sex (%)				0.42 <sup>a</sup>
Male	21 (70.00)	9 (81.82)	12 (63.16)	
Female	9 (30.00)	2 (18.18)	7 (36.84)	
Histopathology (%)				0.62 <sup>a</sup>
Well-differentiated	17 (56.67)	5 (45.45)	12 (63.16)	
Moderate-differentiated	11 (36.67)	5 (45.45)	6 (31.58)	
Poor-differentiated	2 (6.67)	1 (9.09)	1 (5.26)	
Smoking (%)				0.02 <sup>a*</sup>
No	24 (80.00)	6 (54.55)	18 (94.74)	
Yes	6 (20.00)	5 (45.45)	1 (5.26)	
Stage (%)				0.67 <sup>a</sup>
IIA	1 (3.33)	0 (0)	1 (5.26)	
IIIB	9 (30.00)	2 (18.18)	7 (36.84)	
IIIC	16 (53.33)	7 (63.64)	9 (47.37)	
IV	4 (13.33)	2 (18.18)	2 (10.53)	
Pre-CCRT CRM status (%)				<0.001 <sup>a*</sup>
Clear	12 (40.00)	0 (0)	12 (63.16)	
Involved	18 (60.00)	11 (100.00)	7 (36.84)	
Pre-CCRT EMVI (%)				0.64 <sup>a</sup>
Negative	24 (80.00)	8 (72.73)	16 (84.21)	
Positive	6 (20.00)	3 (27.27)	3 (15.79)	
Pre-CCRT CEA (ng/mL), median [IQR]	6.67 [3.13–18.32]	11.00 [3.82–18.31]	6.25 [2.65–19.31]	0.38 <sup>b</sup>
Post-CCRT CEA (ng/mL), median [IQR]	3.34 [2.17–5.44]	4.25 [2.79–9.28]	2.71 [2.14–4.31]	0.07 <sup>b</sup>

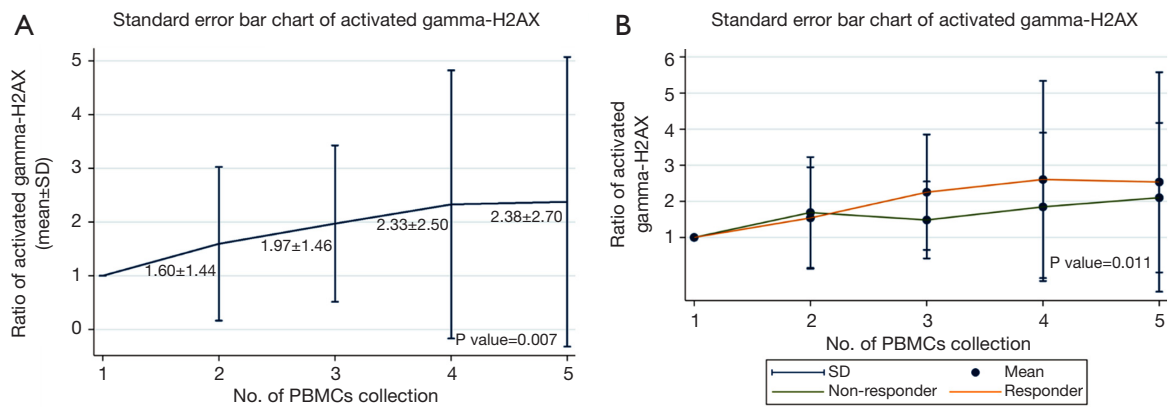
\*, significant at P value of <0.05. <sup>a</sup>, Fisher's exact test. <sup>b</sup>, Mann-Whitney U test. CCRT, concurrent chemoradiation; CEA, carcinoembryonic antigen; CRM, circumferential resection margin; EMVI, extramural vascular invasion; IQR, interquartile range.

0.17 to 1.78,  $P=0.02$ ), the 4<sup>th</sup> (1.33, 95% CI: 0.53 to 2.13,  $P=0.001$ ), and the 5<sup>th</sup> (1.38, 95% CI: 0.57 to 2.18,  $P=0.001$ ) PBMCs collections, as shown in *Figure 4*.

### Pathological outcomes

The median follow-up time for this study was 3.06 years (interquartile range: 2.34–3.43 years). A total of 19 patients (67.86%) underwent oncologic surgery. The remaining 11 patients did not undergo oncologic surgery due to disease progression or distant metastasis in 5 patients, refusal of

surgery in 4 patients, and loss follow-up in 2 patients. The interval between MRI and surgery was 57 days (interquartile range: 38–119 days). During the data collection and patient treatment period, a trend toward adopting TNT emerged. Five patients (26.32%) received chemotherapy with capecitabine and oxaliplatin as part of TNT while waiting for surgery. Four patients (21.06%) achieved a pCR. Additional instances of pathologic downstaging, along with Mandard's five-tier tumor regression grading, were presented in *Table 3*. The association between pCR, non-pCR patients, and  $\gamma$ -H2AX activation was shown in



**Figure 3** Standard error bar chart for ratio of activated  $\gamma$ -H2AX. (A) The increase in activation ratio during treatment was expressed as mean  $\pm$  SD. Repeated measures ANOVA was employed to assess differences between the number of PBMCs collections ( $P=0.007$ ). (B) A higher activation ratio was observed in responders compared to non-responders ( $P=0.011$ ). SD, standard deviation; ANOVA, analysis of variances; PBMCs, peripheral blood mononuclear cells.

**Table 2** Ratio of activated  $\gamma$ -H2AX in non-responders vs. responders based on mrTRG

No. of PBMCs collection	Ratio of activated $\gamma$ -H2AX, mean (SD)			Adjusted difference between groups (95% CI) <sup>#</sup>	P value <sup>#</sup>
	Non-responder	Responder	P value <sup>§</sup>		
2 <sup>nd</sup>	1.70 (1.54)	1.54 (1.41)	0.79	1.17 (-0.02 to 2.34)	0.053
3 <sup>rd</sup>	1.49 (1.07)	2.26 (1.60)	0.46		
4 <sup>th</sup>	1.85 (2.06)	2.61 (2.74)	0.02*		
5 <sup>th</sup>	2.11 (2.08)	2.54 (3.05)	0.19		

<sup>§</sup>, difference of two means using *t*-test. <sup>#</sup>, multilevel linear regression model adjusted for age, sex, smoking, stage, EMVI and CRM status, and histopathology. \*, significant at P value of  $<0.05$ . mrTRG, magnetic resonance tumor regression grade; PBMCs, peripheral blood mononuclear cells; SD, standard deviation; CI, confidence interval; EMVI, extramural vascular invasion; CRM, circumferential resection margin.

Table 4 and Figure 5, similar to the distinction observed between responders and non-responders based on mrTRG. A trend of higher  $\gamma$ -H2AX activation was observed in patients who achieved a pCR compared to those who did not. The differentiation was significant higher in the 4<sup>th</sup> PBMCs collection (5.39 vs. 1.61,  $P=0.02$ ). However, after adjusting for age, sex, smoking status, tumor stage, EMVI, CRM, and histopathology, no statistically significant difference was found ( $P=0.11$ ). Significant difference from 1<sup>st</sup> (95% CI: 0.75 to 3.05,  $P=0.001$ ).

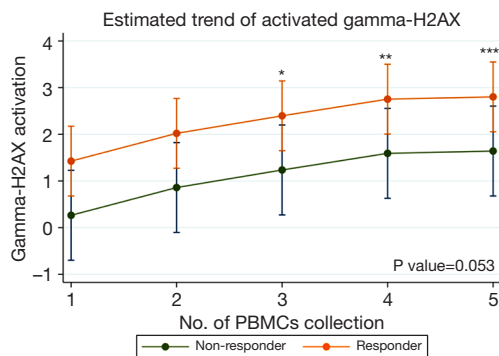
### Survival outcome

The survival outcomes and associations between  $\gamma$ -H2AX activation ratios and survival metrics, including distant metastasis-free survival and overall survival, were analyzed.

The results of these analyses, which did not reveal significant correlations, were detailed in Tables S1,S2.

### Toxicity

The maximal toxicities observed during treatment, based on the CTCAE version 5.0. Skin toxicities were observed in 10 patients, with 8 experiencing grade 1 toxicity and 2 experiencing grade 2 toxicity. Gastrointestinal toxicities were more prevalent, affecting 25 patients, with 6 patients experiencing grade 1 toxicity, 18 experiencing grade 2 toxicity, and only 1 patient experiencing grade 3 toxicity. Genitourinary toxicities were noted in 11 patients, all of whom experienced grade 1 toxicity. Hematologic toxicities were observed in 28 patients, with 20 experiencing grade 1 toxicity and 8 experiencing grade 2 toxicity.



**Figure 4** Marginal prediction plots visualizing trend of activated  $\gamma$ -H2AX between responder and non-responder patients 1.17 (95% CI:  $-0.02$  to  $2.34$ ,  $P=0.053$ ) after adjusted potential confounding factors. \*, significant difference from baseline (1<sup>st</sup>) (95% CI:  $0.17$  to  $1.78$ ,  $P=0.02$ ); \*\*, significant difference from 1<sup>st</sup> (95% CI:  $0.53$  to  $2.13$ ,  $P=0.001$ ); \*\*\*, significant difference from 1<sup>st</sup> (95% CI:  $0.58$  to  $2.18$ ,  $P=0.001$ ). PBMCs, peripheral blood mononuclear cells; CI, confidence interval.

**Table 3** Surgical and pathological outcomes of patients underwent oncologic surgery (N=9)

Surgical outcome	No. of patients (n=19, %)
Pathologic outcome	
ypT0N0 (pCR)	4 (21.06)
ypT1N0	3 (15.79)
ypT2N0	3 (15.79)
ypT3N0	5 (26.32)
ypT3N1	1 (5.26)
ypT3N2	3 (15.79)
ypCRM status	
Clear	18 (94.74)
Involved	1 (5.26)
Mandard's five-tier tumor regression grading (n=13)	
TRG 1	4 (30.77)
TRG 2	1 (7.70)
TRG 3	7 (53.85)
TRG 4	1 (7.70)
TRG 5	0

pCR, pathologic complete response; CRM, circumferential resection margin; TRG, tumor regression grade.

Supplementary analyses were conducted to further explore the relationships between  $\gamma$ -H2AX activation ratios and various hematological parameters, including WBC count and lymphocyte count. Pearson correlation analysis

showed no significant associations between  $\gamma$ -H2AX levels and either WBC count or lymphocyte count across five different time points (refer to Tables S3,S4 for detailed correlation results).

## Discussion

TNT is considered the standard of care for locally advanced rectal cancer patients. The beneficial effect of adding more fluorouracil-based chemotherapy before surgery is to increase the pathological response in patients, especially in patients with poorer responses after receiving CCRT (19,20). Our study used mrTRG to evaluate the response to treatment after CCRT completion. Accordingly, our outcomes were consistent with those of an ongoing TRIGGER study that also used this parameter to further guide treatment for individual patients (21). The radiation technique employed in our treatment protocol was IMRT technique. The volume of the treatment and the dose treatment schedule followed the international consensus guidelines on target volume delineation in rectal cancer (17). The patients who were classified in the responder group was 63.33% in this study. This value is comparable to that of the MERCURY study which reported a value of 48.5% (7).

Apparently,  $\gamma$ -H2AX, a phosphorylated form of histone H2AX, is caused by a DNA double strand break. According to recent data,  $\gamma$ -H2AX has been used to monitor DNA damage that occurs either from chemotherapy or radiation (15,22). Another previous clinical study demonstrated that the mean number of  $\gamma$ -H2AX foci in PBMCs after radiation in breast and rectal cancer patients were significantly enhanced (11,16). However, none of the previous clinical studies demonstrated the dynamic changes during CCRT course.

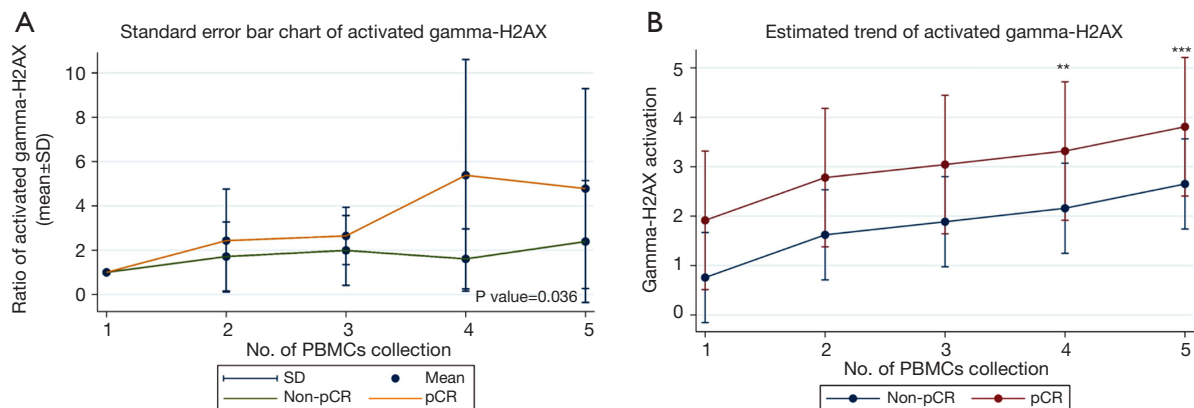
In our study, we collected PBMCs at 5 different time points, from baseline through 6-week after CCRT, and confirmed significant dynamic changes in the ratio of  $\gamma$ -H2AX activation in PBMCs among locally advanced rectal cancer patients during CCRT. After we used the multilevel linear regression model, we found the significantly increased from baseline (1<sup>st</sup>) during CCRT caused and continue to increase to the end. The 2<sup>nd</sup> PBMCs collection did not show a statistically significant difference compared to baseline. This finding may be attributed to the DNA repair process occurring within 24 hours after receiving ionizing radiation. It is consistent with *in vitro* studies demonstrating that the half-life of  $\gamma$ -H2AX is approximately 2 to 7 hours, reflecting the DNA repair process (22,23). After repeated



**Table 4** Ratio of activated  $\gamma$ -H2AX in pathologic complete responders vs. non-responders based on pathologic outcome

No. of PBMCs collection	Ratio of activated $\gamma$ -H2AX, mean (SD)			Adjusted difference between groups (95% CI) <sup>#</sup>	P value <sup>#</sup>
	Non-pCR (n=15)	pCR (n=4)	P value <sup>§</sup>		
2 <sup>nd</sup>	1.72 (1.56)	2.44 (2.33)	0.47	1.16 (−0.25 to 2.56)	0.11
3 <sup>rd</sup>	2.00 (1.58)	2.65 (1.30)	0.46		
4 <sup>th</sup>	1.61 (1.36)	5.39 (5.23)	0.02**		
5 <sup>th</sup>	2.40 (2.75)	4.79 (4.51)	0.19		

<sup>§</sup>, difference of two means using *t*-test. <sup>#</sup>, multilevel linear regression model adjusted for age, sex, smoking, stage, EMVI and CRM status, and histopathology. \*\*, significant at P value of <0.05. PBMCs, peripheral blood mononuclear cells; SD, standard deviation; pCR, pathologic complete response; CI, confidence interval; EMVI, extramural vascular invasion; CRM, circumferential resection margin.



**Figure 5** Ratio of activated  $\gamma$ -H2AX in patients with pCR vs. non-pCR. (A) Standard error bar chart for ratio of activated  $\gamma$ -H2AX in patients with pCR vs. non-pCR. (B) Marginal prediction plots visualizing trend of activated  $\gamma$ -H2AX between pCR and non-pCR. \*\*, significant difference from 1<sup>st</sup> (95% CI: 0.26 to 2.56, P=0.017); \*\*\*, significant difference from 1<sup>st</sup> (95% CI: 0.75 to 3.05, P=0.001). PBMCs, peripheral blood mononuclear cells; SD, standard deviation; pCR, pathologic complete response; CI, confidence interval.

daily radiation fractions during treatment, the cumulative  $\gamma$ -H2AX values in PBMCs increased, showing significantly higher values in the 3<sup>rd</sup> to 5<sup>th</sup> collections.

Two clinical trials investigated the correlation between treatment response and  $\gamma$ -H2AX. Djuzenova *et al.* reported inconsistencies between the number of foci and the response of rectal tumors to treatment (11). In contrast, a study on non-small cell lung cancer found that 67% of patients with a complete response after radiation had consistent  $\gamma$ -H2AX foci numbers during and 12 weeks after treatment (13). The variation in results may be due to differences in the treatment scheme, histopathology, and the time points of assessment. In the present study, patients were categorized as responders or non-responders in this study utilizing the MERCURY system. Our analysis using *t*-test and repeated measure ANOVA revealed that the responders had

significantly higher  $\gamma$ -H2AX values compared to the non-responders. Upon controlling for potential confounding factors, our model also demonstrated that the responder patient exhibited a trend towards increased expression during and after CCRT (P=0.053).

In our additional analysis, 19 patients underwent complete oncologic surgery after preoperative CCRT, with 4 of them (21.06%) showing pCR in the specimen, consistent with findings from a previous trial (19). However, due to the limited data on pathologic TRG, the sample size was too small to perform accurate discrimination. When comparing pCR and non-pCR patients, we observed a differentiation in the  $\gamma$ -H2AX ratio, with an increase in  $\gamma$ -H2AX levels found in patients with pCR, consistent with the responder group using mrTRG data. A significantly higher  $\gamma$ -H2AX ratio was observed in pCR patients during

the 4<sup>th</sup> PBMC collection. Nevertheless, the limited sample size, no significant difference was detected in multilevel linear regression. Furthermore, we did not control the timing of surgery for each patient, and the emerging trend toward TNT during the study may have affected the accuracy of the pathologic response results in categorizing patients.

The study also found that active smokers and involved CRM on diagnostic MRI were statistically significantly related with poor responses to CCRT. In the large meta-analysis of 14 prospective cohort studies, issues of reduced cell-mediated immunity and relative tissue hypoxia were caused by patients who smoked during treatment and were associated with poorer overall survival rates when compared with non-smokers (24,25). In a previous study, CRM was identified as a key prognostic factor in patients with locally advanced disease (26). Notably, there have been limited studies involving CRM and the relevant response after CCRT. Due to the smoking and CRM status correlated with response of treatment, we adjusted these two factors in multilevel linear regression model.

Additionally, we performed a survival analysis. The 2-year overall survival rate in this study was 83.33%, which is comparable to historical data for patients receiving preoperative CCRT (27,28). Most recurrences occurred within the first two years after completing treatment (29). We also analyzed the association between  $\gamma$ -H2AX levels and overall survival, as well as distant metastasis-free survival, but found no significant correlations. This lack of association could be due to the short half-life of  $\gamma$ -H2AX, which may limit its predictive value for long-term outcomes. Key prognostic factors influencing survival included disease stage, tumor grading, performance status, hemoglobin levels, and genetic mutations. To enhance the accuracy of prognostic models, these factors should be incorporated alongside predictive biomarkers (30-32).

Acute toxicity levels that were recorded in our study were within a grade range of 0-2. Only one patient developed acute grade 3 radiation to induce enteritis on the 5<sup>th</sup> week of the CCRT course. Before the follow-up timepoint, this patient presented with immediate large bowel obstruction after CCRT was completed. Diarrhea, which occurred in the later weeks of CCRT, might be considered a retentive encopresis (overflow incontinence). The results of an analysis of the acute treatment related side effects in our study were comparable with those of other retrospective studies using IMRT for preoperative CCRT (33,34). We investigated the correlation between  $\gamma$ -H2AX values and

both WBC count and lymphocyte count. Unfortunately, no significant correlations were identified.

The strength of this study resides in its clinical prospective design. We collected the patient's PMBCs in several timepoints, from baseline to 6-week after CCRT completed, aimed to assess the dynamic change of  $\gamma$ -H2AX overtime. Moreover, we utilized automated flow cytometry techniques to help minimize potential human errors in foci counting. However, certain limitations should be noted. The small sample size prevented the establishment of a definitive cut-off point for the increasing of  $\gamma$ -H2AX. Additionally, the lack of histopathological specimens from TME restricted the depth of our analysis.

## Conclusions

Our study demonstrated the possibility of  $\gamma$ -H2AX as a biomarker for response predictor in patients with locally advanced rectal cancer patients underwent CCRT. There was a trend toward increased expression between responders and non-responders after excluding confounding variables. However, more sample and cut-point level of  $\gamma$ -H2AX research are needed to be explored.

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## Footnote

*Reporting Checklist:* The authors have completed the STROBE reporting checklist. Available at <https://jgo.amegroups.com/article/view/10.21037/jgo-24-488/rc>

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*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related

to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee for Human Research (protocol code RAD-2563-07543, September 25<sup>th</sup>, 2020) at the Faculty of Medicine, Chiang Mai University. Verbal and written informed consent was obtained from all participants after providing them with necessary information.

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