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Associations and prognostic implications of Eastern Cooperative Oncology Group performance status and tumoral LINE-1 Methylation status in stage III colon cancer patients

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Full Title:	Associations and prognostic implications of Eastern Cooperative Oncology Group performance status and tumoral LINE-1 Methylation status in stage III colon cancer patients
Article Type:	Research
Abstract:	Background Low methylation status of LINE-1 in tumors is associated with poor survival in patients with colon cancer. Eastern Cooperative Oncology Group performance status (ECOG-PS) is a method to assess the functional status of a patient. We retrospectively evaluated the relationship between ECOG-PS and LINE-1 methylation in colorectal cancers (CRCs) and their prognostic impact in CRC or colon cancer patients receiving adjuvant 5-fluorouracil/leucovorin/oxaliplatin (FOLFOX). Results LINE-1 methylation and microsatellite instability were analyzed in stage III or high-risk stage II CRCs (n=336). LINE-1 methylation levels were correlated with clinicopathological features, including ECOG-PS and recurrence-free survival (RFS). Association between the tumoral LINE-1 methylation level and ECOG-PS was observed (OR=2.56, P <0.001). Differences in LINE-1 methylation levels in cancer tissue between the ECOG-PS 0 and 1 groups were significant in patients older than 60 years (P=0.001), the overweight BMI group (P=0.005), and the stage III disease group (P=0.008). Prognostic values of LINE-1 methylation status or combined LINE-1 methylation and ECOG-PS statuses were identified in stage III colon cancers, not in stage III or high-risk stage II CRCs. Low LINE-1 methylation status was closely associated with a shorter RFS time. The difference between ECOG(0)/LINE-1(high) and ECOG(1)/LINE-1(low) was significant, which suggests that colon cancer patients with concurrent ECOG(1)/ LINE-1 (low) have a higher recurrence rate. Conclusion ECOG-PS was associated with LINE-1 methylation in CRC tissue. LINE-1 methylation was associated with RFS in patients with stage III colon who were treated with adjuvant FOLFOX chemotherapy. LINE-1 methylation status may improve the ability of ECOG-PS to act as a predictor of recurrence. Further validation and translational studies to improve treatment outcomes in this subset of patients are needed.

Associations and prognostic implications of Eastern Cooperative Oncology Group performance status and tumoral *LINE-1* methylation status in stage III colon cancer patients

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Abstract

Background

Low methylation status of *LINE-1* in tumors is associated with poor survival in patients with colon cancer. Eastern Cooperative Oncology Group performance status (ECOG-PS) is a method to assess the functional status of a patient. We retrospectively evaluated the relationship between ECOG-PS and *LINE-1* methylation in colorectal cancers (CRCs) and their prognostic impact in CRC or colon cancer patients receiving adjuvant 5-fluorouracil/leucovorin/oxaliplatin (FOLFOX).

Results

LINE-1 methylation and microsatellite instability were analyzed in stage III or high-risk stage II CRCs (n=336). *LINE-1* methylation levels were correlated with clinicopathological features, including ECOG-PS and recurrence-free survival (RFS). Association between the tumoral *LINE-1* methylation level and ECOG-PS was observed (OR=2.56, P <0.001). Differences in *LINE-1* methylation levels in cancer tissue between the ECOG-PS 0 and 1 groups were significant in patients older than 60 years (P=0.001), the overweight BMI group (P=0.005), and the stage III disease group (P=0.008). Prognostic values of *LINE-1* methylation status or combined *LINE-1* methylation and ECOG-PS statuses were identified in stage III colon cancers, not in stage III or high-risk stage II CRCs. Low *LINE-1* methylation status was closely associated with a shorter RFS time. The difference between ECOG(0)/*LINE-1*(high) and ECOG(1)/*LINE-1*(low) was significant, which suggests

that colon cancer patients with concurrent ECOG(1)/ *LINE-1* (low) have a higher recurrence rate.

Conclusion

ECOG-PS was associated with *LINE-1* methylation in CRC tissue. *LINE-1* methylation was associated with RFS in patients with stage III colon who were treated with adjuvant FOLFOX chemotherapy. *LINE-1* methylation status may improve the ability of ECOG-PS to act as a predictor of recurrence. Further validation and translational studies to improve treatment outcomes in this subset of patients are needed.

Key words: Adjuvant therapy, colorectal cancer, ECOG, FOLFOX, *LINE-1*, methylation, and prognosis

Background

Colorectal cancer (CRC) is one of the most common cancers and a leading cause of cancer death globally [1]. Despite a declined CRC mortality in developed countries, the incidence and mortality are increasing in East Asian populations, including Korea [2, 3]. Adjuvant chemotherapy significantly decreases mortality in colon cancer, and 5-fluorouracil (5-FU), leucovorin and oxaliplatin (FOLFOX) are the current standard of care for patients with stage III colon cancer after surgery [4, 5]. FOLFOX is also frequently used to treat stage II colon cancer with high-risk features, such as T4 tumors or lymphovascular invasion.

CRC is a multifactorial disease which arises due to the accumulation of genetical and epigenetical alterations. Epigenetic changes are potential factors contributing to the carcinogenesis of colorectal cancer (CRC). Promoter CpG island hypermethylation is associated with the inactivation of tumor suppressors and tumor-related genes [6, 7], and genome-wide hypomethylation is an alternative mechanism for genomic instability, which facilitates tumor progression [8]. Methylation levels of repetitive transposable DNA elements are a useful surrogate marker for global genomic methylation status because repetitive DNA elements reside in intergenic or intronic regions of the genome at extremely high frequencies, and CpG sites located within repetitive DNA elements are usually methylated[9]. Long interspersed nucleotide element-1 (*LINE-1*) is a major constituent of repetitive transposable DNA elements, and it constitutes approximately 17% of the human

genome [9, 10]. *LINE-1* is usually methylated in normal cells, which maintains transcriptional inactivation and inhibits retrotransposition[11]. Hypomethylation of *LINE-1* is a common finding in various tissue types of human cancer [12-18], but hypomethylation of *LINE-1* occurs during premalignant stages of many tissue types of human cancers, including colon, stomach, prostate, and bile duct [15, 19, 16, 20-22, 13].

The Eastern Cooperative Oncology Group - performance status (ECOG-PS) is a global assessment of a cancer patient's actual level of function and self-care ability. Functional levels represent a simple but important clinical tool to predict comorbidities and outcomes, such as response to treatment, duration of response, and survival [23, 24]. ECOG-PS is strongly associated with prognosis in various tissue types of cancer [25-29]. This scale shows high predictive value for medical care requirements, rehabilitation strategies, and prognosis evaluation in cancer patients. However, little is known regarding the relationship between tumoral *LINE-1* methylation levels and ECOG-PS status of cancer patients. The aims of the present study were to verify the possible relationship between tumoral *LINE-1* methylation status and ECOG-PS of CRC patients and to elucidate the prognostic impact of tumoral *LINE-1* methylation status and ECOG-PS in CRC patients treated with adjuvant FOLFOX chemotherapy.

Results

Distribution of clinicopathological parameters in different ECOG groups

A total of 336 patients were included. Clinicopathological parameters of the patients are described in Table 1. ECOG-PS ≥1 was present in 170 (50.5%) patients (169 patients with ECOG-PS 1 and one patient with ECOG-PS 2). The majority of patients were men (n=211, 62.8%), and the average age was 58.9 years (median 60, range 29–78 years). The tumor location was cecum in 9 patients, ascending colon in 75, transverse in 21, descending in 22, sigmoid in 189 and rectum in 20, including 104 right colon carcinomas and 212 left colon carcinomas. T stages 1/2/3/4 were 8/30/255/43 in patients, respectively, and N stages 0/1/2 were 49/203/84 in patients, respectively. Forty-nine patients had high-risk stage II disease (IIA, 33; IIB, 12; IIC, 4), and 287 patients had stage III disease (IIIA, 31; IIIB, 195; IIIC, 61). A total of 165 patients received FOLFOX-4, and 171 patients received modified FOLFOX-6. The distributions of N stage (P=0.032), microsatellite status (P=0.035) and TNR (P=0.002) were different between the two groups, but the other parameters were not (Table 1).

Association between *LINE-1* methylation and clinicopathological parameters

To determine clinicopathological implications of the *LINE-1* methylation status, we analyzed *LINE-1* methylation in cancer and normal lymph node tissue samples. *LINE-1* methylation levels in cancer tissue samples ranged from 29.81% to 78.73%

(median, 52.64%), which was significantly lower than those of normal LN samples which ranged from 48.50% to 89.90% (median, 76.80%, P <0.001). When the ratio of tumor to normal LN *LINE-1* methylation level was described as Tumor to Normal Ratio (TNR), the TNR ranged from 0.63 to 0.78 with median of 0.69.

Association between *LINE-1* methylation levels in cancer tissue samples and the ECOG-PS of the patients was observed (OR=2.56, P <0.001). *LINE-1* methylation levels in normal lymph nodes were also associated with body mass index (BMI) status of the patient (OR=1.76, P=0.015). No association was found between other selected parameters and *LINE-1* methylation levels of cancer tissue or normal lymph node (Table 2).

LINE-1 methylation levels were significantly lower in cancer tissue samples of patients with ECOG-PS≥1 than in those of patients with ECOG-PS 0 (Fig. 1A). However, such a difference was not found in normal LN samples (Fig. 1B). The differences in *LINE-1* methylation in cancer tissues between ECOG-PS 0 and ≥1 were significant in patients aged ≥60 years, overweight patients (BMI ≥23.5), and stage III cancers but not in patients aged <60years, low BMI patients (BMI <23.5), and stage II cancers (Figs. 1C-1E).

ECOG status, *LINE-1* methylation status and recurrence-free survival (RFS)

The median follow-up of the entire cohort at the time of data cut-off for the present analysis was 50.30 months, interquartile range (IQR) 46.68–53.92 months. At last

follow-up, a total of 41 disease-recurrence events (35 distant metastases and 6 local recurrences) were observed. The overall 4.5-year and 9-year RFS estimates were 87.50% and 83.00%, respectively.

LINE-1 methylation was associated with RFS. The optimal cut-off values for *LINE-1* methylation levels were determined as 54.62% which was the median of *LINE-1* methylation levels in the ECOG 0 group. Low *LINE-1* methylation status (< 54.62%) was closely associated with a shorter RFS time. The 4.5-year RFS was 93.9% in the high *LINE-1* methylation patients, and it was 84.9% in the low *LINE-1* methylation patients (log-rank test, P=0.049) (Fig. 2A). There was no significant difference in RFS according to the ECOG status: 4.5-year RFS 93.9% in the ECOG 0 group and 85.7% in the ECOG ≥1 (P=0.248, Fig. 2B). However, multivariate Cox regression analysis revealed that *LINE-1* methylation status was not an independent prognostic parameter (Table 3).

We also performed survival analysis using the TNR value stratified by selected parameters such as age, gender, BMI, tumor location, T stage, N stage and ECOG status. Low TNR value (<0.69) was closely associated with a shorter RFS time in the overweighted patient (higher BMI score). The 4.5-year RFS was 95.6% in the high TNR value patients, and it was 83.8% in the Low TNR value patients (log-rank test, P=0.044). The TNR value was not associated with other selected parameters.

When we counted RFS from the date of chemotherapy to the date of recurrence, Kaplan-Meier log-rank analysis showed survival curves similar to those using RFS

calculated from the date of surgery to the date of recurrence by the Kaplan-Meier method with log-rank test (Supplementary Fig. 1).

Combinatory ECOG and *LINE-1* statuses as a predictor of recurrence in the setting of adjuvant FOLFOX

When we performed survival analysis with combinatory *LINE-1* methylation and ECOG statuses, we found that the 4.5-year RFS was 94.5% in the ECOG(0)/*LINE-1*(high) group (n=93 patients), 91.5% in the ECOG(0)/*LINE-1*(low) group (n=73), 92.1% in the ECOG(1)/*LINE-1*(high) group (n=63) and 81.5% in the ECOG(1)/*LINE-1*(low) group (n=107). The difference was significant between ECOG(0)/*LINE-1*(high) and ECOG(1)/*LINE-1*(low) (pairwise pooled log-rank test, *P* =0.037). We did not perform further analyses of RFS in combination with *BRAF* and *KRAS* status because of the low number of patients with this mutation.

Univariate analyses revealed that higher levels of T stage (HR=2.41, 95% C.I.= 1.179-4.908, *P*=0.016), N stage (HR=2.51, 95% C.I.=1.355-4.654, *P*=0.003) and ECOG(1)/*LINE-1*(low) (HR=2.72, 95% C.I.= 1.013-7.325, *P*=0.047) were associated with higher recurrence. Multivariable Cox regression analyses adjusting for gender, age, BMI, tumor location, histology, T stage, N stage and microsatellite instability status revealed consistent statistical patterns and *P* values in T stage (HR=2.60, 95% C.I.=1.251-5.413, *P*=0.011) and N stage (HR=2.78, 95% C.I.=1.429-5.393, *P*=0.003), but ECOG(1)/*LINE-1*(low) status was not associated with RFS (HR=2.27, 95%

C.I.=0.839-6.168, *P*=0.106) (Table 4).

Prognostic value of *LINE-1* status alone or combined *LINE-1* and ECOG statuses in stage III colon cancers

Because high-risk stage II CRCs or stage III rectal cancers may differ from stage III colon cancers with respect to the beneficial effect from adjuvant FOLFOX vs. 5-FU/ leucovorin, survival analysis was needed to be performed in stage III colon cancers with exclusion of rectal cancers and stage II colon cancers. The associations between *LINE-1* methylation levels with covariates investigated in the study population (stage III colon cancers only) (Table 5) were similar to those between *LINE-1* methylation levels with covariates investigated in the study population (stage III colon cancers plus high-risk stage II CRCs and stage III rectal cancers) (Table 2). Multivariate Cox regression analysis revealed that low *LINE-1* methylation status was associated with a shorter RFS time (Table 6). ECOG(1)/*LINE-1*(low) was independently associated with disease recurrence (HR = 5.06, 95% C.I. = 1.142 - 22.377, *P*=0.033) in multivariate analysis (Table 7).

Discussion

The present study has demonstrated that ECOG-PS was associated with *LINE-1* methylation in cancer tissue. The difference in *LINE-1* methylation in cancer tissue

between ECOG 0 and ECOG≥1 was significant in patients of older age, higher BMI score or stage III disease. *LINE-1* methylation levels change with aging and disease progression, and our stratified analyses of *LINE-1* methylation levels adjusting for potential confounding suggested that low levels of *LINE-1* methylation had a closer relationship with poor ECOG-PS. To our knowledge, the present study is the first study to assess the association between ECOG-PS statuses of CRC patients and *LINE-1* methylation levels of cancer tissue.

Previous studies suggest that *LINE-1* methylation is a strong, independent predictor of overall survival [30]. Our present study also showed that low *LINE-1* methylation status was closely associated with shorter RFS time. However, ECOG-PS was not associated with RFS. This result was not consistent with previous studies showing that PS was predictive of survival in patients with locally advanced or metastatic carcinoma [31, 32]. There are several factors triggering this result. First, this result may be due to the small number of relapse events and the follow-up of RFS. The Kaplan-Meier analysis showed that the curves of ECOG 0 and ECOG≥1 separated early, and most events happened before the median follow-up time, which is an inaccurate measurement of the median RFS. Another reason may be the limited ECOG-PS subgroups in the study. ECOG-PS was distributed into mainly two groups: ECOG 0 and ECOG 1, with only one patient having ECOG 2. The lack of patients with ECOG-PS >1 limited the power to distinguish significant differences in RFS with different ECOG-PS groups. Because the result was not significant in our

condition, we performed statistical power analyses to decide the likelihood that the test can detect effects of a given size in this particular situation. The power analysis found that the overall 336 subjects (170 in ECOG≥1 and 166 in ECOG 0) achieved a 56.93% power at a 0.050 significance level to detect a difference in RFS between ECOG≥1 and ECOG 0 (Ssupplementary Fig. 2A). The sample size estimation suggested that a larger number should be included in further study if the relapse events are rare while the relapse-free proportion stays high during follow-up of the ECOG≥1 group. The stronger power of the precision to provide reliable answers requires larger sample numbers (Supplementary Fig. 2B).

Our results suggested that RFS was highest in patients with ECOG(0)/*LINE-1* (high) and lowest in patients with ECOG(1)/*LINE-1*(low). Therefore, we just found a significant difference between the two groups of patients in univariate analysis. This difference was not significant in multivariate analyses. This lack of significance may be affected by other parameters that are involved in multivariate analysis and the small number of patients in each ECOG/*LINE-1* groups after stratification. However, in stage III colon cancer patients with exclusion of rectal cancers and high risk-stage II colon cancers or rectal cancer patients, not only *LINE-1* methylation status alone but also ECOG(1)/*LINE-1*(low) was independently associated with disease recurrence in multivariate analysis. Whether ECOG(0)/*LINE-1*(low) might identify a subset of patients with poor prognosis is required to be validated with a large-size samples of stage III colon cancers.

The TNR value is more appealing parameter compared with just methylation level of *LINE*-1 measured in cancer tissue samples. The value reflects methylation change in tumor compared with matched normal tissue and removes some artifacts inherent using FFPE samples and methylation analysis technology. It provides possible normalization of many confounding variables in the analysis technology. We found that low TNR value was closely associated with a shorter RFS time in the overweighted patient.

The present study has several limitations. The major limitation was that, because all the patients were treated with adjuvant FOLFOX, we could not evaluate the interaction between *LINE-1* methylation and treatment effect of adjuvant FOLFOX. Nonetheless, our cohort was relatively homogeneous in stage (stage III and high-risk stages II included) and treatment (surgery and adjuvant FOLFOX at a single institution). Another limitation is the relatively short duration of follow-up. However, the median follow-up duration exceeded 4.5 years, and RFS at which point exhibits a good correlation with longer follow-up periods of overall survival (OS) in colon cancer. Further analyses, including determinations of the OS, will be performed in the future after longer durations of follow-up.

Conclusions

We found that ECOG-PS was associated with *LINE-1* methylation in CRC tissue. For stage III colon cancer patients treated with adjuvant FOLFOX, *LINE-1*

methylation is associated with RFS and may serve as a predictor of recurrence. Patients with tumors having concurrent ECOG(1)/*LINE-1*(low) had a higher recurrence rate. Further validation and translational studies to improve treatment outcomes in this subset of patients will be needed in the future. However, *LINE-1* methylation and combined ECOG-PS and *LINE-1* statuses were not prognostic parameters in stage III or high-risk stage II CRCs.

Methods

Patients and Methods

A total of 336 CRC patients who received curative surgery and adjuvant chemotherapy in the Seoul National University Hospital, Seoul, Korea from June 2005 and November 2011 were included. The following eligibility criteria were used for this retrospective study: age at diagnosis > 18 years, stage III (any T and N1 or N2M0) or high-risk stage II (T3 or T4N0M0), completion of at least 6 cycles of adjuvant FOLFOX, and adequate organ functions. High risk was defined as follows: T4 tumor, poor histological grade, lymphovascular invasion, bowel obstruction at presentation and localized perforation. Patients with a distant metastasis or history of other malignancy within the previous 5 years were excluded. Hereditary non-polyposis colon cancer syndrome or familial adenomatous polyposis was excluded. Patients received either FOLFOX-4 (165 patients) or modified FOLFOX-6 (171 patients). Each cycle of FOLFOX-4 consisted of oxaliplatin (85 mg/m^2) on day 1

and folinic acid (200 mg/m^2) and a bolus of 5-FU (400 mg/m^2) followed by a 22-hr infusion of 5-FU (600 mg/m^2) on days 1 and 2, which was repeated every 2 weeks. Modified FOLFOX-6 consisted of oxaliplatin (85 mg/m^2), folinic acid (400 mg/m^2) and a bolus of 5-FU (400 mg/m^2) followed by a 46-hr infusion of 5-FU (2400 mg/m^2) repeated every 2 weeks. Adjuvant chemotherapy was continued until completion of the planned 12 cycles, recurrence, toxicity or patient refusal. In 336 patients, 303 (90.2%) patients had completed all 12 cycles of adjuvant chemotherapy. Thirty three (9.8%) patients completed more than 6 cycles but less than 12 cycles of adjuvant chemotherapy. Computed tomography (CT) imaging was performed every six cycles during the chemotherapy period, and patients were followed up at least every 6 months after completion of the chemotherapy. Recurrence was defined based on CT scans in the case of distant metastasis, and the treating oncologist made the decision to perform pathological confirmation when necessary.

The study protocol was reviewed and approved by the institutional review board of Seoul National University Hospital (1408-075-604). Informed consent was exempted because of the retrospective nature of the study and minimal risk of harm to the study subjects. This study was performed in accordance with the recommendations of the Declaration of Helsinki for biomedical research involving human subjects. Patient records/information was anonymized and de-identified prior to analysis.

Methylation and microsatellite analyses

Analyses of DNA methylation and MSI were performed as described previously [13, 33]. Briefly, tissue slides were reviewed, and areas of high tumor cell density (1 cm^2) were marked and dissected using a knife blade. Normal abdominal lymph node (LN) tissues from the cancer patients were also dissected. Dissected tissues were collected into a microtube containing tissue lysis buffer and proteinase K. The EZ DNA methylation kit was used to convert DNA using sodium bisulfite (Zymo Research, Orange, CA), and *LINE-1* methylation levels were measured using a pyrosequencing methylation assay. We used the same oligonucleotide primers as ones designed by the Issa group [34]. The primers and PCR conditions are listed in Supporting Information Table 1. The *LINE-1* assays were performed in a 25-μL PCR reaction containing 2-μL bisulfite-treated genomic DNA, 60 mM Tris-HCl (pH 8.8), 15 mM ammonium sulfate, 0.5 mM $MgCl_2$, 1 mM dNTP mix, and 1 U of Taq polymerase. The PCR products were purified and quantified in the PyroMark Q24 System (Biotage AB, Uppsala, Sweden). The amounts of C nucleotides relative to the sum of C and T nucleotides at each CpG site were calculated as percentages. The average of two percentage values in the two adjacent CpG sites (nucleotide positions 321 and 306 of X58075 (GenBank)) was taken as the overall *LINE-1* methylation level in a given sample.

The microsatellite status of each tumor was determined using five microsatellite markers (D2S123, D5S346, D17S250, BAT25 and BAT26). Either the forward or reverse primer for each marker was labeled with fluorescence, and PCR products

were electrophoresed and analyzed. We classified MSI status as follows: MSI-high (instability at ≥ 2 microsatellite markers), MSI-low (instability at 1 marker) or MSS (instability at none).

ECOG-PS measurement

PS was measured at the start of chemotherapy using the ECOG-PS, which is an ordinal scale with scores from 0 to 5 [35]: 0, normal activity; 1, symptomatic but ambulatory; 2, symptomatic—confined to bed/chair <50% of waking hours; 3, symptomatic—confined to bed/chair >50% of waking hours; 4, 100% bedridden; and 5, dead. The present study included 166 cases with ECOG-PS 0, 169 cases with ECOG-PS 1, and 1 case with ECOG-PS 2. For all CRC patients, the ECOG-PS scores were rated before initiation of adjuvant therapy.

Statistical analysis

Post-hoc exploratory analysis was performed using individual methylation markers. The number of methylation loci was compared using Student's t-test or one-way analysis of variance (ANOVA). Categorical variables were compared using chi-square test or Fisher's exact test. TNR of *LINE-1* methylation level was calculated by the equation (= (average methylation level at *LINE-1* CpG sites 2 and 3 in tumor) /(average methylation level at *LINE-1* CpG sites 2 and 3 in normal LN)). Unconditional logistic regression analysis was performed to measure the association

between *LINE-1* methylation and clinicopathological parameters. Clinical data were last updated in November 2014. RFS was calculated from the date of surgery to the date of recurrence or death by the Kaplan-Meier method with log-rank test. To adjust for baseline characteristics, we performed univariate and multivariate analysis using a Cox proportional hazard model. Age, gender, stages (II vs. III), histology (mucinous adenocarcinoma vs. others), tumor location (proximal vs. distal) and MSI status (MSI-high vs. others) were included as covariates. The reason why MSI was put into multivariate analysis include 1) that MSI is known to be one of the prognostic biomarkers, 2) that MSI status is associated with tumoral *LINE-1* methylation levels [36], and 3) that survival of patients with MSI-high CRCs depends on tumoral LINE-1 methylation status [37]. Two-sided p-values of <0.05 were considered significant in all analyses. Statistical analyses were performed using SPSS software for Windows, version 17.0 (SPSS, Chicago, IL).

Availability of supporting data

The datasets supporting the results of this article are included within the article.

Competing Interests:

The authors declare that they have no competing interests.

Authors' contributions:

DC, XW and GHK were involved in the study concept and design. DC, XW, YSS, YYR, THL, NYC, SWH, TYK, and GHK involved in the data acquisition. The analysis and interpretation of data was done by DC and XW. The manuscript writing was made by DC and GHK. The manuscript review and final approval were done by DC, XW, YSS, YYR, THL, NYC, SWH, TYK, and GHK. All authors read and approved the final manuscript.

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FIGURE LEGENDS

Fig 1. *LINE-1* methylation levels between ECOG-PS groups. (A, B) *LINE-1* methylation levels are significantly different between ECOG-PS groups in cancer tissues (A) but not normal LN samples (B). (C, D, E) Differences in *LINE-1* methylation in cancer tissue between ECOG-PS groups were significant in patients who were older than 60 years (C), had a BMI indicating overweight (D), and high-risk stage III disease (E).

Fig 2. Survival analyses. (A) Survival analyses show that low *LINE-1* methylation status was closely associated with shorter RFS times (p=0.049). (B) There was no significant difference in RFS according to the ECOG status. (C) Survival analysis stratified by status combined with ECOG-PS and *LINE-1*. The difference was significant between ECOG(0)/*LINE-1*(high) and ECOG(1)/*LINE-1*(low) (p=0.037). (D) Survival analysis using the TNR value in higher BMI score patients.

Table 1. Baseline characteristics of the epidemiological and clinical variables of the study population

Parameters	ECOG performance status			
	Case N (%)	0 N (%)	≥ 1[a] N (%)	*P*[b]*-value*
Gender				
Female	125 (37.2)	64 (38.6)	61 (35.9)	0.612
Male	211 (62.8)	102 (61.4)	109 (64.1)	
Age (Median)				
<60 years	165 (49.1)	90 (54.2)	75 (44.1)	0.064
≥60 years	171 (50. 9)	76 (45.8)	95(55.9)	
BMI				
Normal	135 (40.2)	74 (44.6)	61 (35.9)	0.104
Overweight	201 (59.8)	92 (55.4)	109 (64.1)	
Tumor location				
Proximal	105 (31.3)	54 (32.5)	51 (30.0)	0.617
Distal	231 (68.7)	112 (67.5)	119 (70.0)	
Pathology				
Non-mucinous carcinoma	321 (95.5)	160 (96.4)	161 (94.7)	0.599[c]
Mucinous adenocarcinoma	15 (4.5)	6 (3.6)	9 (5.3)	
Differentiation				
low grade	311 (92.6)	156 (94.0)	155 (91.2)	0.328
high grade	25 (7.4)	10 (6.0)	15 (8.8)	
T stage				
T1, 2, 3	293 (87.2)	147 (88.6)	146 (85.9)	0.464
T4	43 (12.8)	19 (11.4)	24 (14.1)	
N stage				
N 0, 1	252 (75.0)	133 (80.2)	119 (70.0)	0.032
N 2	84 (25.0)	33 (19.9)	51 (30.0)	
Microsatellite status				
MSS + MSI-L	313 (93.1)	149 (89.8)	164 (96.5)	0.035
MSI-H	21 (6.3)	15 (9.0)	6 (3.5)	
Missing	2 (0.6)	2 (1.2)	0	
TNR (Median)				
<0.69	173 (51.5)	71 (42.8)	102 (60.0)	0.002
≥0.69	163 (48.5)	95 (57.2)	68 (40.0)	

Abbreviations: BMI, Body Mass Index; ECOG, Eastern Cooperative Oncology Group; MSS, Microsatellite stable; MSI-L, Microsatellite instability-low; MSI-H, Microsatellite instability-high; TNR, Tumor to Normal Ratio.

[a] ECOG ≥1 group including one patient with ECOG 2; the rest had ECOG 1.

[b]Chi-square test or [c]Fisher's exact test.

Table 2. Binary logistic regression analyses of the association of *LINE-1* methylation and covariates investigated according to the ECOG-PS-dependent variable in the study population (stage III and high-risk stage II CRCs)

Parameters	*LINE-1* methylation levels (low *vs.* high)[a]			
	Cancer sample		Normal LN sample	
	OR	*P* [b] (95% C.I.)	OR	*P* [b] (95% C.I.)
Gender	Ref.		Ref.	
(Male vs. Female)	1.49	0.104 (0.921-2.406)	1.06	0.803 (0.670-1.677)
Age	Ref.		Ref.	
(≥60 years vs. <60 years)	1.20	0.450 (0.752-1.899)	0.95	0.823 (0.611-1.480)
BMI	Ref.		Ref.	
(Overweight vs. Normal)	1.24	0.366 (0.775-1.996)	1.76	0.015 (1.118-2.765)
Tumor location	Ref.		Ref.	
(Distal vs. Proximal)	0.81	0.429 (0.474-1.373)	1.26	0.370 (0.762-2.079)
Pathology	Ref.		Ref.	
(Mucinous vs. Non-mucinous)	0.83	0.802 (0.196-3.521)	1.44	0.616 (0.349-5.926)
Differentiation	Ref.		Ref.	
(high grade vs. low grade)	0.37	0.100 (0.111-1.211)	0.68	0.515 (0.213-2.173)
T stage	Ref.		Ref.	
(T4 vs. T1, 2, 3)	0.81	0.555 (0.403-1.629)	0.68	0.255 (0.347-1.324)
N stage	Ref.		Ref.	
(N2 vs. N 0, 1)	1.24	0.443 (0.715-2.156)	1.15	0.602 (0.681-1.938)
Microsatellite status	Ref.		Ref.	
(MSI-H vs. MSS+MSI-L)	0.33	0.052 (0.106-1.008)	0.76	0.580 (0.283-2.026)
ECOG status	Ref.		Ref.	
(ECOG ≥1 vs. ECOG=0)	2.56	<0.001 (1.601-4.102)	1.12	0.615 (0.718-1.752)

Abbreviations: BMI, Body Mass Index; ECOG, Eastern Cooperative Oncology Group; MSS, Microsatellite stable; MSI-L, Microsatellite instability-low; MSI-H, Microsatellite instability-high.

[a]Cut-off points of *LINE-1* methylation proportion were 54.62% in cancer tissue samples and 77.00% in normal LN samples.

[b]Unconditional logistic regression, adjusted for other selected covariates.

Table 3. Univariate and multivariate Cox regression analyses of ECOG-PS with disease recurrence

Parameters	Multivariate analysis in stage III or high-risk stage II CRCs		
	HR	95% C.I.	*P* [a]
Gender (Male vs. Female)	1.74	0.848-3.557	0.131
Age (≥60 years vs. <60 years)	1.38	0.725-2.628	0.326
BMI (Overweight vs. Normal)	1.27	0.653-2.475	0.481
Tumor location (Distal vs. Proximal)	1.92	0.831-4.458	0.127
Pathology (Mucinous vs. Non-mucinous)	2.00	0.285-14.074	0.485
Differentiation (high grade vs. low grade)	0.66	0.130-3.369	0.619
T stage (T1, 2, 3 vs. T4)	2.68	1.289-5.589	0.008
N stage (N 0, 1 vs. N2)	2.80	1.437-5.441	0.002
Microsatellite status (MSI-H vs. MSS+MSI-L)	2.43	0.659-8.981	0.182
ECOG Status ECOG(1)/ ECOG(0)	1.14	0.579-2.233	0.710
LINE-1 methylation *LINE-1* (low)/ *LINE-1* (high)	2.07	0.932-4.607	0.074

Abbreviations: BMI, Body Mass Index; ECOG, Eastern Cooperative Oncology Group; MSS, Microsatellite stable; MSI-L, Microsatellite instability-low; MSI-H, Microsatellite instability-high.

[a]Cox proportional hazard regression model, adjusted for other selected covariates.

Table 4. Univariate and multivariate Cox regression analyses of ECOG-PS with disease recurrence (stage III and high-risk stage II CRCs)

Parameters	Univariate analysis			Multivariate analysis		
	HR	95% C.I.	*P*	HR	95% C.I.	*P* [a]
Gender (Male vs. Female)	1.66	0.834-3.321	0.149	1.70	0.831-3.489	0.146
Age (≥60 years vs. <60 years)	1.45	0.774-2.719	0.245	1.33	0.698-2.546	0.383
BMI (Overweight vs. Normal)	1.29	0.674-2.454	0.445	1.24	0.634-2.427	0.529
Tumor location (Distal vs. Proximal)	1.94	0.897-4.205	0.092	1.87	0.808-4.320	0.144
Pathology (Mucinous vs. Non-mucinous)	1.29	0.311-5.343	0.726	1.95	0.277-13.789	0.502
Differentiation (high grade vs. low grade)	1.15	0.353-3.718	0.821	0.65	0.127-5.413	0.602
T stage (T4 vs. T1, 2, 3)	2.41	1.179-4.908	0.016	2.60	1.251-5.413	0.011
N stage (N 2 vs. N 0, 1)	2.51	1.355-4.654	0.003	2.78	1.429-5.393	0.003
Microsatellite status (MSI-H vs. MSS+MSI-L)	1.24	0.381-4.002	0.725	2.43	0.656-8.996	0.184
ECOG Status_ *LINE-1* methylation						
ECOG(0)/*LINE-1* (high)	Ref.					
ECOG(0)/*LINE-1* (low)	2.02	0.684-5.936	0.203	1.91	0.638-5.704	0.248
ECOG(1)/*LINE-1* (high)	1.25	0.360-4.337	0.343	1.12	0.320-3.917	0.859
ECOG(1)/*LINE-1* (low)	2.72	1.013-7.325	0.047	2.27	0.839-6.168	0.106

Abbreviations: BMI, Body Mass Index; ECOG, Eastern Cooperative Oncology Group; MSS, Microsatellite stable; MSI-L, Microsatellite instability-low; MSI-H, Microsatellite instability-high.

[a]Cox proportional hazard regression model, adjusted for other selected covariates.

Table 5. Binary logistic regression analyses of the association of *LINE-1* methylation and covariates investigated according to the ECOG-PS-dependent variable in the study population (stage III colon cancers only)

Parameters	*LINE-1* methylation levels (low *vs.* high)[a]			
	Cancer sample		Normal LN sample	
	OR	*P* [b] (95% C.I.)	OR	*P* [b] (95% C.I.)
Gender	Ref.		Ref.	
(Male vs. Female)	1.63	0.065 (0.971-2.747)	1.11	0.670 (0.699-1.745)
Age	Ref.		Ref.	
(≥60 years vs. <60 years)	1.23	0.419 (0.742-2.053)	0.97	0.893 (0.624-1.508)
BMI	Ref.		Ref.	
(Overweight vs. Normal)	1.21	0.487 (0.711-2.044)	1.56	0.055 (0.991-2.444)
Tumor location	Ref.		Ref.	
(Distal vs. Proximal)	0.78	0.393 (0.438-1.383)	1.14	0.598 (0.694-1.887)
Pathology	Ref.		Ref.	
(Mucinous vs. Non-mucinous)	0.77	0.759 (0.149-4.004)	1.52	0.569 (0.361-6.387)
Differentiation	Ref.		Ref.	
(high grade vs. low grade)	0.58	0.420 (0.154-2.185)	0.51	0.270 (0.154-1.687)
T stage	Ref.		Ref.	
(T1, 2, 3 vs. T4)	0.99	0.985 (0.417-2.359)	0.74	0.382 (0.381-1.447)
N stage	Ref.		Ref.	
(N 0, 1 vs. N2)	1.07	0.812 (0.600-1.921)	1.21	0.470 (0.720-2.039)

Microsatellite status	Ref.		Ref.	
(MSI-H vs. MSS+MSI-L)	0.40	0.150 (0.118-1.388)	1.02	0.972 (0.380-2.728)
ECOG status	Ref.		Ref.	
(ECOG ≥1 vs. ECOG=0)	1.80	0.024 (1.081-3.004)	1.20	0.421 (0.770-1.871)

Abbreviations: BMI, Body Mass Index; ECOG, Eastern Cooperative Oncology Group; MSS, Microsatellite stable; MSI-L, Microsatellite instability-low; MSI-H, Microsatellite instability-high.

[b]Unconditional logistic regression, adjusted for other selected covariates.

Table 6. Univariate and multivariate Cox regression analyses of ECOG-PS with disease recurrence

Parameters	Multivariate analysis in stage III colon cancer only		
	HR	95% C.I.	*P* [a]
Gender (Male vs. Female)	1.86	0.796-4.360	0.151
Age (≥60 years vs. <60 years)	1.32	0.629-2.779	0.461
BMI (Overweight vs. Normal)	2.09	0.878-4.980	0.096
Tumor location (Distal vs. Proximal)	2.73	0.978-7.624	0.055
Pathology (Mucinous vs. Non-mucinous)	3.13	0.384-25.534	0.286
Differentiation (high grade vs. low grade)	0.77	0.140-4.176	0.757
T stage (T1, 2, 3 vs. T4)	2.96	1.235-6.993	0.014
N stage (N 0, 1 vs. N2)	2.40	1.133-5.085	0.022
Microsatellite status (MSI-H vs. MSS+MSI-L)	2.19	0.341-13.989	0.409
ECOG Status ECOG(1)/ ECOG(0)	1.29	0.597-2.800	0.516
LINE-1 methylation *LINE-1* (low)/ *LINE-1* (high)	3.16	1.181-8.430	0.022

Abbreviations: BMI, Body Mass Index; ECOG, Eastern Cooperative Oncology Group; MSS, Microsatellite stable; MSI-L, Microsatellite instability-low; MSI-H, Microsatellite instability-high.

[a]Cox proportional hazard regression model, adjusted for other selected covariates.

Table 7. Univariate and multivariate Cox regression analyses of ECOG-PS with disease recurrence (stage III colon cancers only)

Parameters	Univariate analysis			Multivariate analysis		
	HR	95% C.I.	*P*	HR	95% C.I.	***P*** [a]
Gender (Male vs. Female)	1.96	0.881-4.367	0.099	1.85	0.792-4.327	0.155
Age (≥60 years vs. <60 years)	1.54	0.775-3.161	0.234	1.36	0.642-2.876	0.423
BMI (Overweight vs. Normal)	2.23	0.966-5.164	0.060	2.22	0.907-5.413	0.081
Tumor location (Distal vs. Proximal)	2.32	0.955-5.637	0.063	2.74	0.984-7.608	0.054
Pathology (Mucinous vs. Non-mucinous)	1.71	0.409-7.166	0.462	3.06	0.376-24.961	0.295
Differentiation (high grade vs. low grade)	1.41	0.428-4.619	0.575	0.78	0.143-4.228	0.771
T stage (T1, 2, 3 vs. T4)	2.97	1.284-6.865	0.011	3.05	1.282-7.249	0.012
N stage (N 0, 1 vs. N2)	2.40	1.196-4.795	0.014	2.43	1.147-5.127	0.020
Microsatellite status (MSI-H vs. MSS+MSI-L)	1.06	0.254-4.451	0.933	2.16	0.339-13.813	0.415
ECOG Status_ *LINE-1* methylation						
ECOG(0)/ *LINE-1* (high)	Ref.					
ECOG(0)/ *LINE-1* (low)	3.69	0.794-17.167	0.096	4.40	0.912-21.200	0.065
ECOG(1)/ *LINE-1* (high)	1.78	0.297-10.678	0.528	2.08	0.333-12.970	0.434
ECOG(1)/ *LINE-1* (low)	5.20	1.197-22.611	0.028	5.06	1.142-22.377	0.033

Abbreviations: BMI, Body Mass Index; ECOG, Eastern Cooperative Oncology Group; MSS, Microsatellite stable; MSI-L, Microsatellite instability- low; MSI-H, Microsatellite instability-high.

[a]Cox proportional hazard regression model, adjusted for other selected covariates.

Supporting Information

Supplementary Table 1. Primer sequences and PCR conditions used for pyrosequencing

LINE-1	Primer	Tm (℃)
Forward	5′-TTTTGAGTTAGGTGTGGGATATA-3′	**52**
Reverse	5′-biotin-AAAATCAAAAAATTCCCTTTC-3′	
Sequencing	5′-AGTTAGGTGTGGGATATAGT-3′	

S1 Fig. Survival analyses using RFS from the date of chemotherapy. (A) Survival analyses show that low *LINE-1* methylation status was closely associated with shorter RFS times (p=0.040). (B) There was no significant difference in RFS according to the ECOG status. (C) Survival analysis stratified by status combined with ECOG-PS and *LINE-1*. The difference was significant between ECOG(0)/*LINE-1*(high) and ECOG(1)/*LINE-1*(low) (p=0.021). (D) Survival analysis using the TNR value in higher BMI score patients.

S2 Fig. The power and sample size analysis. (A) Overall, 336 subjects (170 in ECOG 1 and 166 in ECOG 0) achieved 56.93% power at a 0.050 significance level to detect differences RFS between ECOG 1 and ECOG 0. (B) The sample size soars as power or ECOG 1 relapse-free proportion increases.

Figure 1
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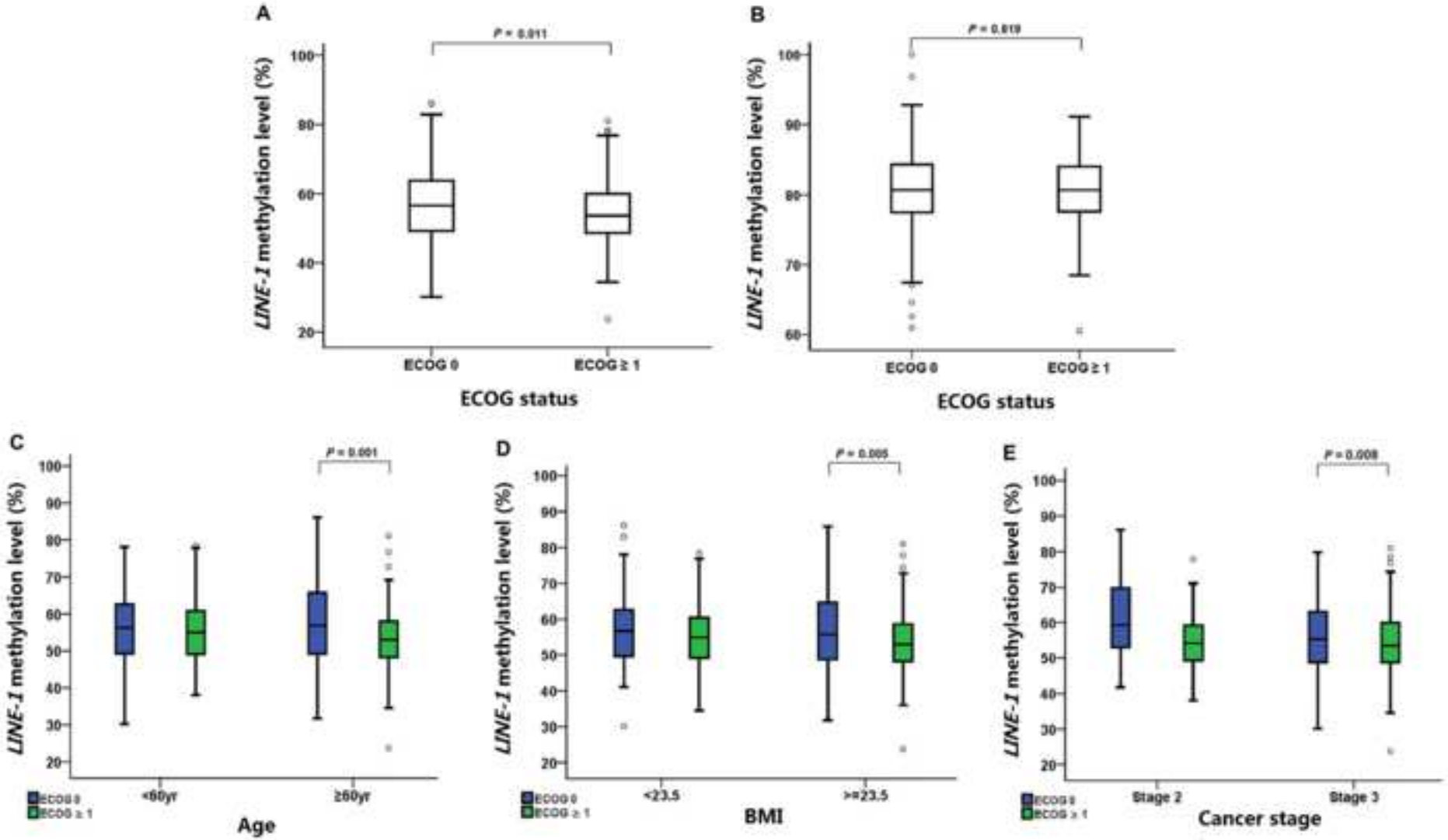

Figure 2
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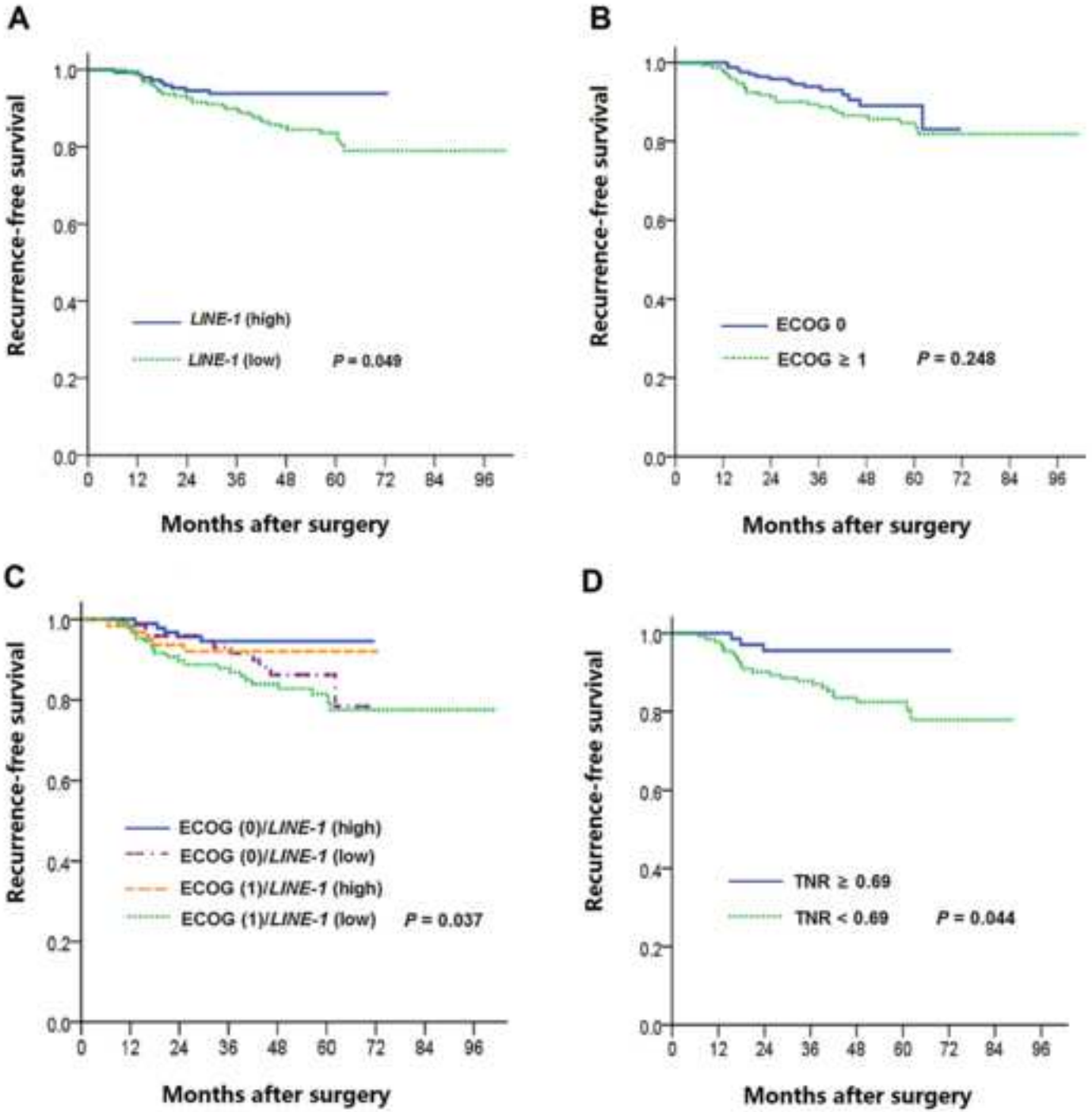

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