Preoperative detection of KRAS mutated circulating tumor DNA is an independent risk factor for recurrence in colorectal cancer

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Preoperative ctDNA status in relation to recurrence in cases of CRC remains unclear. We examined preoperative ctDNA detection by targeting KRAS gene mutations as a predictive marker for recurrence after CRC surgery. We measured the preoperative KRAS mutated ctDNA status and analyzed the correlation with clinicopathologic features of 180 patients that underwent surgery for CRC. We studied the association between preoperative KRAS mutated ctDNA and postoperative recurrence in patients (n = 150) that underwent radical surgery. KRAS mutated ctDNA was detected in 59 patients (32.8%). Median mutant allele frequency of KRAS in ctDNA was 0.20%. KRAS status in ctDNA and lymph node metastasis and distant metastasis were not significantly different. Among patients that underwent radical resection, recurrence occurred in 21 (14.0%, median follow-up 24 months). In Kaplan–Meier analysis, preoperative detection of KRAS mutated ctDNA was associated with inferior recurrence-free interval (RFI) (p = 0.002) and recurrence-free survival (RFS) (p = 0.025). In a multivariate Cox proportional hazards model, preoperative detection of KRAS mutated ctDNA was an independent factor related to both RFI (HR = 3.08; p = 0.012) and RFS (HR = 2.18; p = 0.044). Preoperative measurement of KRAS mutated ctDNA could be useful to decide postoperative treatment.

Colorectal cancer (CRC) is one of the most common types of cancer. It is important to identify predictive factors for recurrence to manage CRC patients. Adjuvant chemotherapies have been used in stage III CRC and stage II cases judged to have a high risk of recurrence based on clinicopathologic features, such as poorly differentiated tumor, vascular, lymphatic or perineural invasion, tumor depth of T4, lymph nodes sampling < 12, or clinical presentation with intestinal occlusion or perforation1–6. CRC treatment outcomes have improved due to recent advances in medical technology, but determining treatment strategies for postoperative recurrence is often difficult.

In recent years, circulating tumor DNA (ctDNA) has attracted attention as a predictor of postoperative recurrence. It is a fraction of cell-free DNA (cfDNA), which is derived from cancer cells and contains tumor specific DNA mutations7–9. ctDNA levels in plasma are typically low, but recent technological advancements, such as digital droplet PCR (ddPCR) and next generation sequencing (NGS) platforms, have enabled detection of ctDNA in frequencies as low as 0.01%10. The presence of postoperative circulating tumor DNA (ctDNA) has been demonstrated to be associated with recurrence after CRC surgery11–13. However, the relationship in CRC between preoperative ctDNA status and recurrence is unclear. Meanwhile, the presence of preoperative ctDNA has been reported to be associated with recurrence in other cancers, such as pancreatic cancer and breast cancer14–16. The detection rate of ctDNA has been shown to decrease after surgery14, so examining preoperative ctDNA status may help to distinguish more cases considered to be at high risk of recurrence.

In CRC, KRAS gene is mutated in about 40% of cases, mostly appearing in segment in exon 2 (codon 12 and 13). Point mutations in the KRAS gene have been reported to occur early in the carcinogenic process and are detected at the same frequency in tissue biopsy, regardless of the cancer stage17,18. KRAS exon 2 mutations have been reported to be associated with recurrence and poor prognosis after CRC surgery18–20. Therefore, we focused on KRAS mutations in ctDNA, but not ctDNA with other mutations. ctDNAs have various mutations including KRAS, BRAF, NRAS, PIK3CA and so on. It has been shown that KRAS and BRAF mutations are mutually exclusive18,21. ctDNA without KRAS mutation may contain BRAF mutation such as V600E.

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In this study, we measured the preoperative KRAS mutated ctDNA and evaluated any association with clinicopathologic factors. We also evaluated the usefulness of preoperative KRAS mutated ctDNA detection as a predictive marker of recurrence after CRC surgery.

**Results**

**Mutant and wild-type KRAS in cfDNA detected by ddPCR.** Mutant and wild-type KRAS ctDNA were shown separately by the Quantasoft software Ver1.7.4 (Bio-Rad), such as shown in Fig. 1. The ratio of positive drops for the mutant and/or the wild-type allele was calculated as mutant allele frequency (MAF), and 0.02% was set as the lower limit of ctDNA detection, as previously reported.

**KRAS mutation in ctDNA and lymph node and distant metastasis.** Among 180 patients, 59 were positive for KRAS mutation in ctDNA (32.8%). The median MAF of KRAS in ctDNA was 0.20% (range 0.04–68.99%). Patient demographic data and KRAS status in ctDNA by clinicopathologic factors are shown in Table 1. The frequency of KRAS mutation positive in ctDNA was higher in cases with histological types other than well-differentiated, in cases with invasion depth of T3 or T4, cases with lymphatic invasion, cases with venous invasion, and stage IV cases. However, multivariate analysis did not reveal significant difference in these factors. Subsequently, we investigated the relationship between KRAS status in ctDNA and lymph node metastasis and with distant metastasis (Table 2). Factors such as invasion depth of T3 or T4, lymphatic invasion, venous invasion and positivity for preoperative serum CEA had correlation to lymph node metastasis. Regarding KRAS status in ctDNA, cases with preoperative KRAS mutation positive for ctDNA tended to have lymph node metastasis and distant metastasis. Multivariate analysis showed venous invasion was significantly related to lymph node metastasis ($p = 0.011$), but there were no significant differences between KRAS status in ctDNA and lymph node metastasis or distant metastasis (Table 3).

![Figure 1. Detection of KRAS mutated ctDNA by ddPCR.](image-url)
We prospectively examined the association between preoperative KRAS status in ctDNA and recurrence in 150 cases, excluding 26 cases with synchronous distant metastasis, three cases without distant metastasis who could not undergo radical resection, and one case of hospital death due to pneumonia and renal failure a month after surgery. Recurrence was diagnosed by imaging examinations. Regarding adjuvant chemotherapy, capecitabine single agent or capecitabine plus oxaliplatin was generally given to stage III and high risk stage II CRC patients in our department. In this study, the use of adjuvant chemotherapy was decided by the treating oncologist who was blinded to the ctDNA status. Capecitabine single agent was given to 32 patients and capecitabine plus oxaliplatin was given to 19 patients. Fifteen of 38 ctDNA positive patients and 36 of the 73 ctDNA negative patients received adjuvant chemotherapy in stage II and III CRC patients.

Among the 150 patients with stage III or lower stage CRC that underwent radical resection, recurrence occurred in 21 patients (14.0%), including 12 of the 45 patients with detectable KRAS mutations in ctDNA (26.7%) and 9 of the 105 patients without such mutations (8.6%). Fifteen of the 150 patients (10.0%) died before median follow-up of 24 months (range 12–37 months). Patients with preoperative detectable KRAS mutations in ctDNA had an increased risk of recurrence relative to those without them \((p = 0.003)\). Kaplan–Meier analysis showed that preoperative detection of KRAS mutated ctDNA was associated with inferior recurrence-free interval \((RFI) (p = 0.002)\) (Fig. 2a) and recurrence-free survival \((RFS) (p = 0.025)\) (Fig. 2b). In a multivariate Cox proportional hazards model, preoperative detection of KRAS mutated ctDNA was the significant factor correlated to both RFI \((HR = 3.08; p = 0.012)\) and RFS \((HR = 2.18; p = 0.044)\) (Tables 4 and 5).

**Preoperative KRAS mutation in ctDNA and recurrence.** We prospectively examined the association between preoperative KRAS status in ctDNA and recurrence in 150 cases, excluding 26 cases with synchronous distant metastasis, three cases without distant metastasis who could not undergo radical resection, and one case of hospital death due to pneumonia and renal failure a month after surgery. Recurrence was diagnosed by imaging examinations. Regarding adjuvant chemotherapy, capecitabine single agent or capecitabine plus oxaliplatin was generally given to stage III and high risk stage II CRC patients in our department. In this study, the use of adjuvant chemotherapy was decided by the treating oncologist who was blinded to the ctDNA status. Capecitabine single agent was given to 32 patients and capecitabine plus oxaliplatin was given to 19 patients. Fifteen of 38 ctDNA positive patients and 36 of the 73 ctDNA negative patients received adjuvant chemotherapy in stage II and III CRC patients.

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**Discussion**

KRAS mutation in CRC primary lesions were reported to be associated with a high risk of postoperative recurrence.\(^{18-20}\) We demonstrated that preoperative KRAS mutation in ctDNA is an independent risk factor for recurrence in patients with CRC. Although KRAS mutation in ctDNA may be detected in plasma without malignancy, it is extremely rare. Hence, preoperative measurement of KRAS mutation in ctDNA could reflect the primary lesion\(^{23,24}\). The concordance rate of KRAS status between cancer tissue and ctDNA has been reported as 75–96%\(^{25-27}\), because the measurement of gene mutations in tissue has a problem of heterogeneity. Meanwhile a previous study of pancreatic cancer showed that mutant KRAS in plasma was significantly associated with recurrence and prognosis, but not in tumor tissue samples\(^{27}\). The measurement of KRAS mutation in ctDNA or in primary tumors may reflect different aspects of CRC.

![Table 1. Clinicopathologic variables for KRAS status in ctDNA. Well well differentiated adenocarcinoma; others moderately differentiated, poorly differentiated, mucinous and papillary adenocarcinoma.](https://www.nature.com/scientificreports/)

| Variable                  | Total (N = 180) | Positive (N = 59) | Negative (N = 121) | p value |
|---------------------------|-----------------|-------------------|--------------------|---------|
| Age                       |                 |                   |                    |         |
| > 66                      | 133             | 45                | 88                 | 0.611   |
| < 66                      | 47              | 14                | 33                 |         |
| Gender                    |                 |                   |                    |         |
| Male                      | 100             | 33                | 67                 | 0.943   |
| Female                    | 80              | 26                | 54                 |         |
| Tumor site                |                 |                   |                    |         |
| Colon                     | 140             | 45                | 95                 | 0.734   |
| Rectum                    | 40              | 14                | 26                 |         |
| Differentiation           |                 |                   |                    |         |
| Well                      | 57              | 12                | 45                 | 0.027   |
| Others                    | 122             | 46                | 76                 |         |
| Depth                     |                 |                   |                    |         |
| T3, 4                     | 125             | 49                | 76                 | 0.006   |
| T1, 2                     | 55              | 10                | 45                 |         |
| Lymphatic invasion        |                 |                   |                    |         |
| Present                   | 58              | 25                | 33                 | 0.036   |
| Absent                    | 117             | 32                | 85                 |         |
| Venous invasion           |                 |                   |                    |         |
| Present                   | 87              | 37                | 50                 | 0.005   |
| Absent                    | 88              | 20                | 68                 |         |
| Stage                     |                 |                   |                    |         |
| Stage ≤ III               | 154             | 46                | 108                | 0.043   |
| Stage IV                  | 26              | 13                | 13                 |         |
| Preoperative serum CEA    |                 |                   |                    |         |
| ≥ 5.0                     | 71              | 29                | 42                 | 0.063   |
| < 5.0                     | 109             | 30                | 79                 |         |
In recent years, several reports have demonstrated "postoperative" detectable ctDNA as a predictive factor for recurrence\(^1\)\(^-\)\(^3\). However, the detection rate of ctDNA has been reported to decrease after surgery. Reinert et al. reported that in stage I-III CRC, the detection rate of ctDNA decreased from 88.5% preoperatively to 10.6% postoperatively\(^13\). If ctDNA mutations in "postoperative" plasma samples after radical resection can be detected, it may indicate remnant cancer cells resulting in recurrence and poor prognosis. However, if patient has a small amount of cancer cells after radical surgery, ctDNA mutations could not be detected. Preoperative KRAS mutations in ctDNA reflecting mutations of primary lesion may present malignant phenotype of primary tumor. Therefore, we analyzed "preoperative" ctDNA mutations, but not postoperative mutations in the current study. A comparison between preoperative and postoperative ctDNA may provide more information regarding recurrence and prognosis. Further investigation is needed to address this issue. In addition, preoperative ctDNA positive cases were reported to have a high risk of recurrence in localized pancreatic cancer, even if postoperative

**Table 2.** Univariate analysis of lymph node metastasis and distant metastasis. *Well* well differentiated adenocarcinoma; *others* moderately differentiated, poorly differentiated, mucinous and papillary adenocarcinoma.

| Variable         | Lymph node metastasis | Distant metastasis |
|------------------|------------------------|--------------------|
|                  | Present | Absent | \(p\) value | Present | Absent | \(p\) value |
| Age              |          |        |           |          |        |           |
| ≥ 66             | 60      | 73     | 0.340     | 21      | 112    | 0.388     |
| < 66             | 25      | 22     |           | 5       | 42     |           |
| Gender           |          |        |           |          |        |           |
| Male             | 43      | 57     | 0.205     | 11      | 89     | 0.142     |
| Female           | 42      | 38     |           | 15      | 65     |           |
| Tumor site       |          |        |           |          |        |           |
| Colon            | 63      | 77     | 0.264     | 21      | 119    | 0.692     |
| Rectum           | 22      | 18     |           | 5       | 35     |           |
| Differentiation  |          |        |           |          |        |           |
| Well             | 19      | 38     | 0.013     | 7       | 50     | 0.657     |
| Others           | 65      | 57     |           | 18      | 104    |           |
| Depth            |          |        |           |          |        |           |
| T3, 4            | 73      | 52     | <0.001    | 25      | 100    | 0.001     |
| T1, 2            | 12      | 43     |           | 1       | 54     |           |
| Lymphatic invasion|        |        |           |          |        |           |
| Present          | 38      | 20     | <0.001    | 12      | 46     | 0.013     |
| Absent           | 42      | 75     |           | 9       | 108    |           |
| Venous invasion  |          |        |           |          |        |           |
| Present          | 56      | 31     | <0.001    | 17      | 70     | 0.002     |
| Absent           | 24      | 64     |           | 4       | 84     |           |
| Serum CEA        |          |        |           |          |        |           |
| ≥5.0             | 45      | 26     | <0.001    | 19      | 52     | <0.001    |
| <5.0             | 40      | 69     |           | 7       | 102    |           |
| KRAS mutated ctDNA|        |        |           |          |        |           |
| Positive         | 34      | 25     | 0.051     | 13      | 46     | 0.043     |
| Negative         | 51      | 70     |           | 13      | 108    |           |

**Table 3.** Multivariate analysis of lymph node metastasis and distant metastasis. *HR* hazard ratio; *CI* confidence interval; *others* moderately differentiated, poorly differentiated, mucinous and papillary adenocarcinoma; *CEA* carcinoembryonic antigen.

| Variable          | Lymph node metastasis | Distant metastasis |
|-------------------|------------------------|--------------------|
|                   | HR 95% CI | \(p\) value | HR 95% CI | \(p\) value |
| Differentation (others) | 1.55  | 0.71–3.39 | 0.271 | – | – |
| Depth (T3, 4)      | 2.16  | 0.93–5.01 | 0.074 | 4.09 | 0.47–35.90 | 0.204 |
| Lymphatic invasion | 2.05  | 0.98–4.26 | 0.055 | 1.81 | 0.67–4.91 | 0.244 |
| Venous invasion    | 2.59  | 1.25–5.37 | 0.011 | 2.33 | 0.67–8.06 | 0.182 |
| Serum CEA (≥5.0)   | 1.63  | 0.80–3.34 | 0.180 | 2.29 | 0.82–6.40 | 0.114 |
| KRAS mutated ctDNA | 1.11  | 0.54–2.30 | 0.772 | 1.68 | 0.63–4.47 | 0.301 |
Table 4. Univariate and multivariate analysis of recurrence-free interval. HR hazard ratio; CI confidence interval; Well well differentiated adenocarcinoma; CEA carcinoembryonic antigen; CCI Charlson comorbidity index.

| Variable             | Univariate analysis | Multivariate analysis |
|----------------------|---------------------|-----------------------|
|                      | HR 95% CI           | p value               | HR 95% CI           | p value               |
| Age (≥ 66)           | 1.32 0.52–4.06      | 0.576                 |                      |                      |
| Gender (female)      | 1.57 0.66–3.77      | 0.304                 |                      |                      |
| Tumor site (colon)   | 1.77 0.60–7.59      | 0.329                 |                      |                      |
| Differentiation (well) | 1.07 0.41–2.58     | 0.878                 |                      |                      |
| Depth (T3, 4)        | 2.33 0.86–1.16      | 0.101                 |                      |                      |
| Lymphatic invasion   | 1.70 0.69–4.03      | 0.237                 |                      |                      |
| Venous invasion      | 2.83 1.17–7.52      | 0.021                 | 1.50 0.59–4.19       | 0.400                 |
| Lymph node metastasis| 5.20 2.03–15.91     | < 0.001               | 4.06 1.53–12.76      | 0.004                 |
| Serum CEA (≥ 5.0)    | 2.44 1.03–5.99      | 0.044                 | 1.68 0.70–4.21       | 0.245                 |
| CCI (≤ 1)            | 1.46 0.42–9.18      | 0.591                 |                      |                      |
| Adjuvant chemotherapy| 1.40 0.57–3.33      | 0.447                 |                      |                      |
| KRAS mutated ctDNA   | 3.58 1.51–8.79      | 0.004                 | 3.08 1.29–7.63       | 0.012                 |

Table 5. Univariate and multivariate analysis of recurrence-free survival. HR hazard ratio; CI confidence interval; Well well differentiated adenocarcinoma; CEA carcinoembryonic antigen; CCI Charlson comorbidity index.

| Variable             | Univariate analysis | Multivariate analysis |
|----------------------|---------------------|-----------------------|
|                      | HR 95% CI           | p value               | HR 95% CI           | p value               |
| Age (≥ 66)           | 1.90 0.78–5.65      | 0.168                 |                      |                      |
| Gender (female)      | 1.63 0.78–3.49      | 0.195                 |                      |                      |
| Tumor site (colon)   | 1.76 0.68–5.99      | 0.267                 |                      |                      |
| Differentiation (well) | 1.19 0.53–2.52     | 0.668                 |                      |                      |
| Depth (T3, 4)        | 2.01 0.86–5.45      | 0.109                 |                      |                      |
| Lymphatic invasion   | 1.08 0.46–2.32      | 0.856                 |                      |                      |
| Venous invasion      | 1.83 0.87–3.97      | 0.114                 |                      |                      |
| Lymph node metastasis| 2.47 1.17–5.44      | 0.018                 | 2.38 1.13–5.25       | 0.023                 |
| Serum CEA (≥ 5.0)    | 1.59 0.74–3.37      | 0.226                 |                      |                      |
| CCI (≤ 1)            | 0.92 0.36–3.14      | 0.884                 |                      |                      |
| Adjuvant chemotherapy| 1.04 0.46–2.20      | 0.929                 |                      |                      |
| KRAS mutated ctDNA   | 2.28 1.07–4.80      | 0.034                 | 2.18 1.02–4.61       | 0.044                 |

Figure 2. Kaplan–Meier analysis of recurrence-free interval (a), and recurrence-free survival (b), according to preoperative KRAS status in ctDNA.
ctDNA becomes negative\textsuperscript{15}. We showed that preoperative KRAS mutation in ctDNA is associated with recurrence of CRC. Preoperative detection of KRAS mutated ctDNA may provide adequate postoperative screening and appropriate postoperative adjuvant chemotherapy. In the current study, the significance of preoperative ctDNA measurement to determine the indication for adjuvant chemotherapy was not clarified due to a small number of patients. Clinical trial is needed to address this issue.

Circulating tumor DNA is a novel means of detecting early phase CRC recurrence. Postoperative detection of ctDNA in stage II or III CRC reflected minimal residual disease and predicted recurrence\textsuperscript{11,12}. It was measured in these reports by NGS, but clinical application was very costly. ddPCR has been reported to measurable at a lower cost and in a shorter time, yet with higher sensitivity than NGS\textsuperscript{26}. In the current study, KRAS mutated ctDNA measured by ddPCR was significantly correlated with recurrence of CRC and was an independent risk factor for recurrence of CRC.

Our study has several important limitations; only a comparatively small number of patients that had recurrent CRC were included, and the study was of explorative design and there was no validation cohort. Further investigations are required to address these issues.

In conclusion, the presence of KRAS mutated ctDNA before surgery was significantly associated with recurrence after radical resection in cases of CRC. Preoperative KRAS mutated ctDNA measurement was suggested to be a potentially useful biomarker to predict postoperative recurrence. Recurrence may be reduced by administering adjuvant chemotherapy to ctDNA positive patients.

Methods

Patients. In this study, we investigated the relationship between KRAS status in ctDNA, lymph node metastasis, distant metastasis and clinicopathologic factors including age, gender, tumor site, differentiation, tumor depth, lymphatic invasion, venous invasion, preoperative serum CEA value, co-morbidity and adjuvant chemotherapy. In order to consider the effect of co-morbidity on recurrence and prognosis, we evaluated by using the Charlson Comorbidity Index (CCI)\textsuperscript{28,29}.

Enrolled in this study were 183 patients with CRC that underwent surgery at the Second Department of Surgery, Wakayama Medical University, between April 2017 and December 2018. We excluded patients that received preoperative treatment, such as chemotherapy, radiotherapy or endoscopic resection. We also excluded cases diagnosed with other primary cancers and cases with other tumors found by preoperative imaging examinations.

In addition, in three cases, surgery was performed for preoperative clinical diagnosis of CRC, and the patients were not diagnosed with adenocarcinoma by postoperative pathological diagnosis, and these were also excluded from statistical analyses. All research was performed in accordance with relevant guidelines/regulations. This study was approved by the Wakayama Medical University Human Ethics Review Committee (Approval Number 1949) and informed consent was obtained from all included patients.

Blood sample collection and extraction of cfDNA. Just before the start of surgery, 5 mL blood samples were obtained in EDTA tubes from each patient and centrifuged at 1900g for 10 min within 2 h after collection. Plasma was collected and stored at – 80 °C until use. After thawing plasma samples, they were centrifuged at 16,000g for 10 min. cfDNA was extracted from 2 mL of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer’s instructions. Samples were eluted in 75 µL elution buffer and cfDNA was frozen at – 80 °C until analysis. Blood for CEA was collected at the first visit and measured within 2 h.

Detection of KRAS mutated ctDNA. KRAS mutations in ctDNA was analyzed by ddPCR. QX200 Droplet Digital PCR system (Bio-Rad) and ddPCR KRAS multiplex assays including G12A, G12C, G12D, G12R, G12S, G12V, G13D mutations (Bio-Rad) were used according to the manufacturer’s protocols. A reaction volume of 20 µL including 8 µL of cfDNA was used as a template for each PCR. Droplets were generated using the QX200 droplet generator (Bio-Rad) and PCR reaction was performed in a C1000 Touch Thermo Cycler (Bio-Rad) under the following conditions: 95 °C for 10 min, 40 cycles of 94 °C for 30 s and 55 °C for 1 min, and 98 °C for 10 min. Data analysis were performed using the Quantasoft software Ver1.7.4 (Bio-Rad).

Statistics. Statistical analysis was performed using JMP ver. 14.1.0 (SAS Institute). Differences between groups were determined using Pearson’s chi-squared test to compare categorical variables as appropriate. Factors with \( p < 0.10 \) on univariate analysis were analyzed by multivariate logistic regression, and an odds ratio with a 95% confidence interval was calculated for each factor. The Kaplan–Meier method was used to estimate recurrence-free interval (RFI) and recurrence-free survival (RFS), and the log-rank test was used to determine the statistical significance. Cox proportional hazards model was used to assess the risk ratio under simultaneous contributions from several covariates. Final statistical results were considered significant at \( p < 0.05 \).

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Received: 24 August 2020; Accepted: 15 December 2020
Published online: 11 January 2021

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Author contributions

Y.N. performed all experiments and statistical analysis, and wrote the manuscript. S.Y. conceived and designed the study, and gave approval for the final write up. K.M., K.T., Y.M., H.I., Y.M., and D.M. performed surgery and collected specimens. Y.K. provided advice about the experiments and writing the manuscript. H.Y. helped to design the study and draft the manuscript. All authors read and approved the final manuscript.

Funding

Grant-in-Aid 23591904 from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Competing interests

The authors declare no competing interests.
Additional information
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