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Transcriptome biomarkers of colon cancer liver metastasis response to neoadjuvant triplet chemotherapy: a case series

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ABSTRACT

Introduction: The triplet FOLFOXIRI (fluorouracil, leucovorin, oxaliplatin, and irinotecan) may be considered an effective option in the neoadjuvant setting for metastatic colon cancer (mCRC). To investigate potential molecular criteria for treatment response, we evaluate the transcriptome of paired primary colon tumors and liver metastases.

Method: Two sets of quadruple-matched specimens (primary colon tumor, liver metastasis, normal colon and liver tissues) from five patients with resectable mCRC before and after neoadjuvant FOLFOXIRI were selected for RNA sequencing (RNA-seq).

Results: RNA-seq data showed that liver metastases exhibited a higher number of differentially expressed genes (DEGs) than colon tumors (FDR < 0.05, 301 vs. 62, respectively). Up-regulation of *IL1RN, MTCO1P12, RN7SL1, ALDH1A1, DUSP1, COX1*, and *FOS* may be associated with colon tumor sensitivity to FOLFOXIRI. *HBB, GADD45B, DUSP1, FOSB, HBA2, TSC22D3, TAGLN, PER1, CSRP1, CCN2, NAMPT, ZBTB16, SERPINE1, ISG20, SRGN, ATF3, IL7R, IFITM2*, and *KLF2* may potentially be involved in the partial liver metastasis response. *EPS8L2* was the only gene highly expressed in pre-treatment liver tissue of the complete responder patient compared to others (|Log2FC| = 3.84, FDR < 0.05).

Conclusion: Data obtained indicate transcriptional discordance between the primary tumors and liver metastases during neoadjuvant FOLFOXIRI, with the pattern of DEGs involved in their response being distinct. The EPS8L2 transcript could be regarded as a candidate biomarker of liver complete response; however, prognostic conclusions cannot be drawn from this cohort.

Keywords: Colon cancer liver metastasis, Metastatic colon cancer, Neoadjuvant FOLFOXIRI, RNA-seq, Transcriptome biomarkers

Introduction

Initially, metastatic colon cancer (mCRC) accounts for up to 30% of all colorectal cancers with a highly heterogeneous nature (1). Colon cancer distant metastases are observed in multiple sites; however, the liver is the only site of distant disease in one third of mCRC patients (2). Current NCCN and ESMO guidelines establish multimodal treatment, including surgery, chemotherapy, targeted therapy, and immunotherapy as the standard care for resectable mCRC (1,3).

FOLFOXIRI is a combination of three cytotoxic agents, such as fluorouracil, leucovorin, oxaliplatin, and irinotecan, that are considered in the neoadjuvant setting for resectable mCRC according to consensus statements (1,3). In the initial GONO clinical trial, neoadjuvant FOLFOXIRI improved response rates, progression-free survival, and overall survival compared with FOLFIRI (fluorouracil, leucovorin, and irinotecan) with manageable toxicity in unresectable mCRC patients (4). Subsequent randomized trials confirmed that 6 months of induction treatment with FOLFOXIRI provides a clinically

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relevant improvement in 5-year survival compared with 6 months of induction treatment with FOLFIRI (5). Recent studies suggest that neoadjuvant FOLFOXIRI has a favorable safety profile and down-staging effect on locally advanced resectable rectal cancer (6).

The combination of FOLFOXIRI with anti-vascular endothelial growth factor antibody, such as bevacizumab, in patients with unresectable mCRC was investigated by several trials and demonstrated improved survival and response rates compared with doublet chemotherapy regimens (7,8). The addition of cetuximab, an anti-epidermal growth factor receptor antibody, to triplet chemotherapy also showed encouraging results (9,10).

It is important to note that the use of neoadjuvant chemotherapy with FOLFOXIRI for resectable mCRC remains undocumented. However, potential molecular criteria for choosing the optimal treatment regimen that would significantly improve patient outcomes have not been developed. Here we report a transcriptome of primary colon tumors and their matched liver metastatic lesions that were associated with treatment response in a group of mCRC patients who were treated with neoadjuvant FOLFOXIRI plus targeted drug.

Materials and methods

Patient Selection

The prospective study enrolled 5 patients with resectable mCRC who received neoadjuvant treatment in combination with a targeted agent at the Tomsk Cancer Research Institute between 2021 and 2022. All patients were males aged between 40 and 66 years. The inclusion criteria were as follows: resectable mCRC with liver-only metastases, pathologically confirmed adenocarcinoma, Eastern Cooperative Oncology Group performance status of 0-2, and known *RAS* status. In all patients, the primary tumor was located on the left side of the colon. Patients with more than one primary tumor and deficient mismatch repair, or a high microsatellite instability phenotype, were excluded. The patient characteristics are listed in Table 1.

Treatment regimens and response evaluation

All patients received 3 cycles of neoadjuvant FOLFOXIRI chemotherapy followed by synchronous or staged colectomy and liver metastases resection. FOLFOXIRI consisted of irinotecan 165 mg/m^2 on day 1, oxaliplatin 85 mg/m^2 on day 1, leucovorin 200 mg/m^2 on days 1 and 2, and 3200 mg/m^2 48 h continuous infusion of 5-fluorouracil on days 1 and 2. A combination regimen with FOLFOXIRI and cetuximab (500 mg/m^2 on day 1) was given to 2 patients. Three patients received the FOLFOXIRI-only regimen. After surgical resection, all patients received three cycles of adjuvant treatment with FOLFOXIRI within 6 weeks.

Tumor response was assessed by contrast-enhanced computed tomography (CT) of the chest, abdomen and pelvis or contrast-enhanced magnetic resonance imaging (MRI) of the pelvis according to RECIST (version 1.1) guidelines. All 5 patients (100.0%) achieved a partial response of the primary colon tumor. Four of them (80.0%) also had a partial response of metastatic liver lesions, and one patient (20.0%) had a complete response of metastatic lesions. Additional metastatic lesions assessment by intraoperative ultrasound (IOUS) was performed to confirm the complete liver response. Pathological response evaluation of colon tumor and liver metastasis was assessed by the Mandard grading system (11). No serious adverse events (grades III–IV) occurred.

Patient Specimens

A first set of biopsy specimens, including colon tumor and liver metastasis, as well as normal colon and liver tissue, was obtained from each patient before treatment (pre-treatment specimens). The second set included the same specimen's type obtained after surgical resection of both the primary lesion and metastasis (post-treatment specimens). However, one patient had a complete response of the metastatic focus in the liver and, thus, there was an incomplete second specimen set. The tumor cell content in the studied specimens was at least 40%. Normal tissue specimens were obtained at a distance of at least 0.5 cm from the tumor. All specimens were placed in RNAlater solution (Ambion, USA), incubated for 24 hours at +4°C and stored at –80°C.

RNA Isolation and Sequencing

Two sets of quadruple-matched specimens (primary colon carcinoma, liver metastases, normal colon and liver tissues) from each patient before and after treatment were selected for high-throughput RNA sequencing (RNA-seq). Total RNA was extracted using a PureLink RNA Mini Kit (Invitrogen, USA). Sequencing libraries were prepared using the MGIEasy rRNA Depletion V1.3 (MGI, China). RNA-seq library was sequenced on the DNBSeq G400 (MGI, China), using DNBSEQ-G400RS High-throughput Sequencing Kit (MGI, China).

TABLE 1 - Patient characteristics

Case	Specimen sets	Age at diagnosis, years	Sex	Primary tumor site	Histopathology type	Targeted therapy	Response colon tumor/ liver metastasis
1	Complete	42	Male	Rectosigmoid	Mode	No	Partial/Partial
2	Complete	62	Male	Rectosigmoid	Well	Cetuximab	Partial/Partial
3	Complete	39	Male	Rectosigmoid	Mode	No	Partial/Partial
4	Complete	53	Male	Rectosigmoid	Mode	Cetuximab	Partial Partial
5	Incomplete	66	Male	Rectosigmoid	Well	No	Partial/Complete

Statistical analysis

Quality control of sequencing was assessed using FastQC, QoRTs and MultiQC software Online. The DEGs were analyzed by the software package DESeq2. Adjusted P values/false discovery rates (FDR) were calculated using the Benjamini–Hochberg procedure (12). Data visualized by Phantasus Online as well as by the R 4.0.2 package Online.

A Kaplan Meier plotter Online tool was used to examine the relapse-free survival of colon cancer patients for the validation of *EPS8L2* (13). The analysis of *EPS8L2* expression was focused on a cohort of stage 3 and 4 colon cancer patients with left-sided tumors, stable or low microsatellite phenotype based on GEO, EGA and TCGA databases. The hazard ratio with 95% confidence intervals and log-rank test were used to compare differences among survival curves. A p-value of less than 0.05 was considered statistically significant.

Results

DEGs association with primary colon tumor response

In total, 62 DEGs (FDR < 0.05) between the pre-treatment and post-treatment colon specimens were identified (Fig. 1A). The top 20 up-regulated genes of pre-treatment tumors included the *CCR4, FER1L4, AMH, RHOV, GALT, CELSR3, DRAM1, RHPN1, PKMYT1, PCSK9, GRM8, MMP11, WDR62, ALG8, PABPC1L, DNM1, TMEM132A, ARHGAP39, CDH3*, and *MELTF* (|Log2FC|>1.00, FDR<0.05). Only seven up-regulated genes were found in the post-treatment colon tumors, namely *IL1RN, MTCO1P12, RN7SL1, ALDH1A1, DUSP1, COX1*, and *FOS* (|Log2FC| > 1.00, FDR<0.05; Fig. 1B). Given the partial response of the primary colon tumor in all patients, the up- or down-regulation of these genes identified after the treatment may be implicated in the colon tumor sensitivity to FOLFOXIRI alone or in combination with targeted drug.

DEGs association with partial liver metastasis response

Similar to the primary colon tumors, the transcriptome profile of liver metastases was also modulated by neoadjuvant treatment. Cluster analysis revealed a broader range of DEGs (FDR < 0.05, 301 vs. 62) compared to colon tumors (Fig. 1C). We selected the top 20 up-regulated genes *MUC3A, CDCA7, SATB2, XPNPEP2, EPCAM, SCNN1A, TONSL, AIFM3, ENTPD8, GAL3ST2, EPPK1, ANO9, SPIRE2, PTK6, SLC17A1, IQANK1, DGAT1, WNK2, FAM83E,* and *TJP3* that were in the pre-treatment liver tissue (|Log2FC| > 1.00, FDR<0.05). *HBB, GADD45B, DUSP1, FOSB, HBA2, TSC22D3, TAGLN, PER1, CSRP1, CCN2, NAMPT, ZBTB16, SERPINE1, ISG20, SRGN, ATF3, IL7R, IFITM2*, and *KLF2* were up-regulated in the post-treatment liver lesions (|Log2FC| > 1.00, FDR<0.05; Fig. 1D). Since all patients had metastatic liver lesions that responded to treatment, the detected transcripts may also be potentially associated with the efficacy of FOLFOXIRI used alone or in combination with cetuximab.

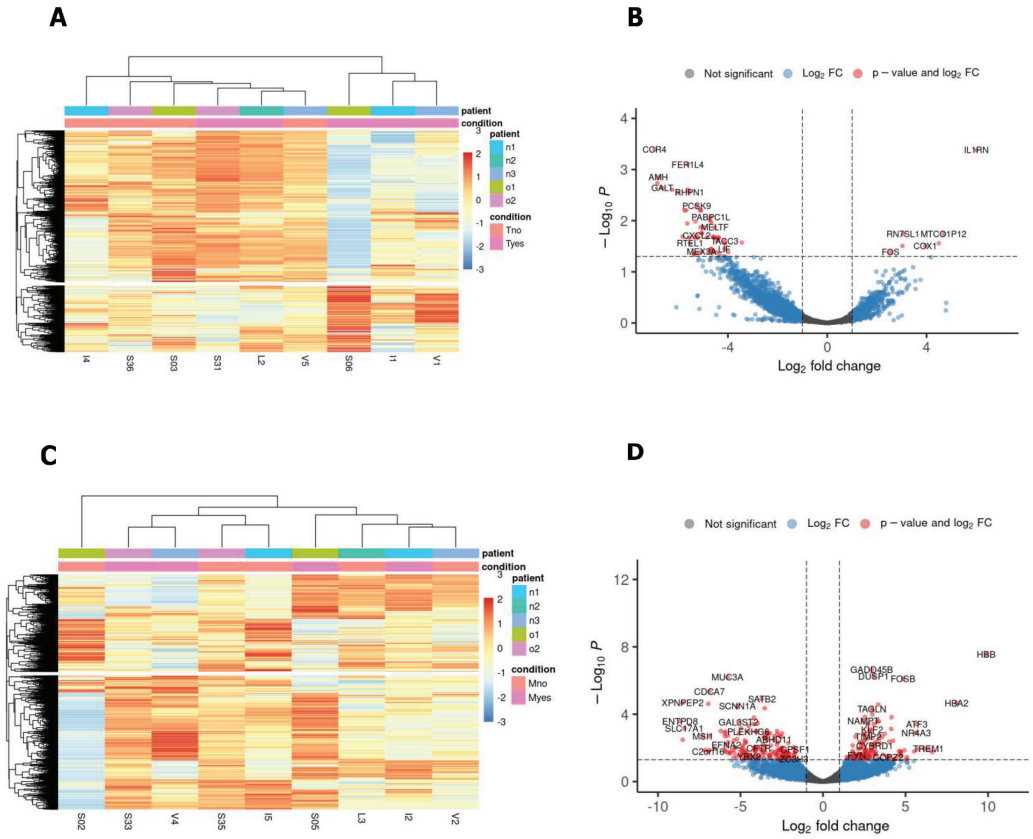

FIGURE 1 - Comparative transcriptome of primary colon tumors and matched liver metastases in mCRC patients during neoadjuvant FOLFOXIRI. Cluster analysis of differentially expressed genes (DEGs) between the pre-treatment and post-treatment colon specimens. Up-regulated genes are represented in red, and down-regulated genes are represented in blue (A); Volcano plot for DEGs in primary colon tumors (B); Cluster analysis of DEGs between the pre-treatment and post-treatment liver lesions. Up-regulated genes are represented in red, and down-regulated genes are represented in blue (C); Volcano plot for DEGs in liver metastases (D).

DEGs association with complete liver metastasis response

To identify genes predicting complete liver metastasis response after FOLFOXIRI, we performed additional transcriptome analysis in a patient who achieved a complete regression of liver metastatic lesions. Interestingly, *EPS8L2* was the only highly expressed in this patient's liver tissue before treatment compared to other patients (|Log2FC| = 3.84, FDR<0.05; Fig. 2A).

In addition, according to a public data set, low *EPS8L2* expression was associated with better relapse-free survival in colon cancer patients (HR = 3.41, 95% CI = 0.92–12.68, Log rank p = 0.052, Fig. 2B). The encoded protein is thought to be involved in regulating actin cytoskeleton remodeling. These observations suggest that *EPS8L2* expression can be modulated by FOLFOXIRI, and subsequent inhibition of cell migration may contribute to metastasis-suppressing function and increased sensitivity to therapy.

Discussion

In this study, we attempted to identify biomarkers associated with FOLFOXIRI response by comparing the transcriptome of primary colon tumors and their matched liver metastatic lesions in mCRC patients. Our data suggested that the use of neoadjuvant FOLFOXIRI, either as a single agent or with cetuximab, had a significant impact on gene expression changes in both primary tumor and metastases.

Previous studies have mainly examined the molecular changes at the genomic level that occur during doublet first-line systemic therapy in mCRC patients. In particular, the comparison of the copy number aberration landscape in liver metastases before and after chemotherapy revealed genomic variations that have a direct impact on the transcriptome (14). A pilot Japanese study indicated that the mutation rate and mutation spectrum were nearly identical regardless of FOLFOX therapy in the four recurrent colorectal cancer cases (15). However, some gene amplifications were observed only in the pre- and post-FOLFOX metastasis specimens compared to the primary tumor, suggesting that copy number variations can change during tumor progression. A recent multicenter prospective biomarker study, REVEAL, supported the differentially expressed gene data between pre-therapeutic primary tumor and post-therapeutic liver metastasis using a Nanostring assay with a 770 cancer-related gene panel (16). Although large mCRC case numbers treated with different chemotherapy regimens were recruited in this study, including six patients who received FOLFOXIRI with either bevacizumab or panitumumab, the impact of each regimen on the

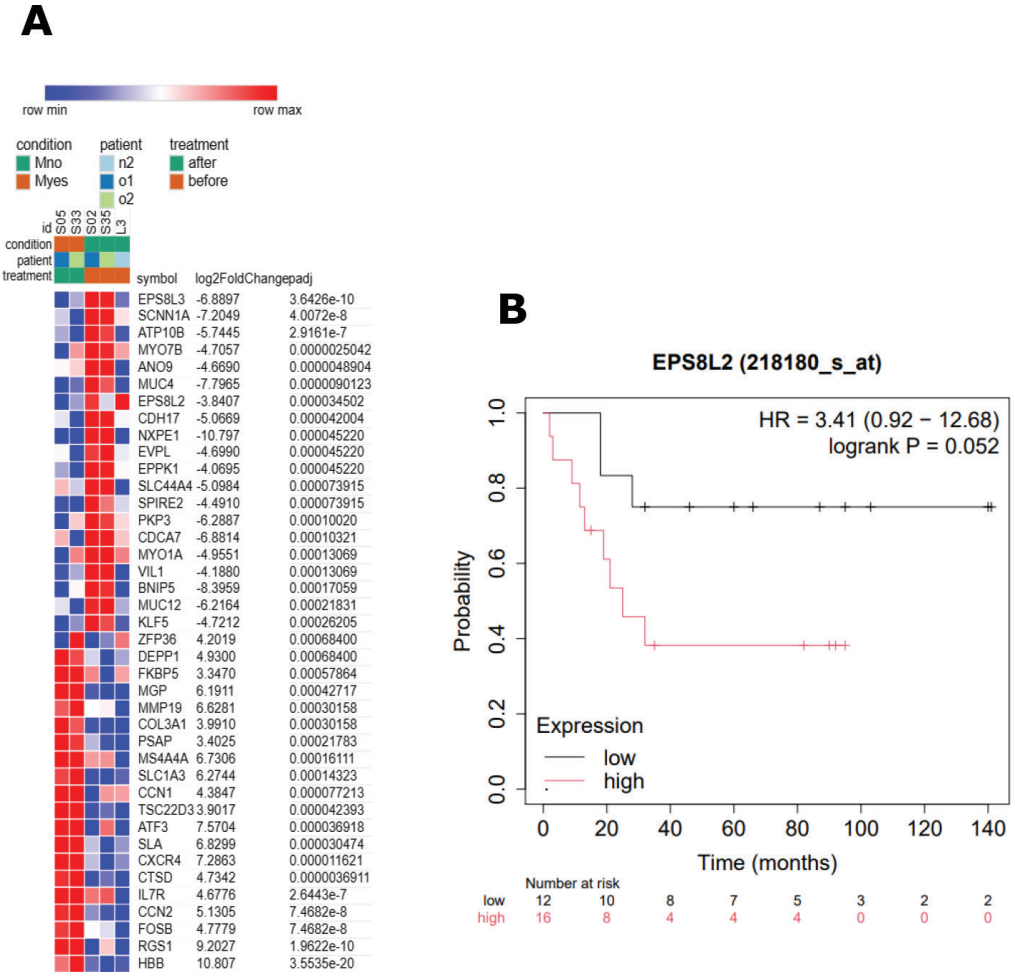

FIGURE 2 - Comparative transcriptome of liver metastases associated with complete response and validation of *EPS8L2* expression based on Kaplan Meier plotter. Heatmap with differentially expressed genes (DEGs) in a patient with complete response of liver metastatic lesions (A); Relapse-free survival stratified by *EPS8L2* expression for colon cancer patients (B).

gene expression signature was not assessed (16). In contrast to the REVEAL study, our results indicate transcriptional differences between primary tumors and liver metastases during neoadjuvant FOLFOXIRI, with the DEGs pattern changing between paired pre- and post-treatment specimens.

We identified an EPS8L2 transcript that could potentially be associated with the complete liver metastasis response and mCRC prognosis. It was recently known to be involved in nuclear movement and cell migration (17). Furthermore, there is clear evidence of the epidermal growth factor receptor pathway substrate 8 (Eps8) involvement in drug response in cell lines (18,19), and it is possible that EPS8L2 will also play a role in regulating drug resistance. The correlation between *EPS8L2* expression and patient survival based on publicly available data suggests its potential utility as a prognostic marker.

It is important to note a number of limitations of this study. Despite the prospective data set, we were able to analyze only a small number of cases. Also, the lack of non-responders in our cohort limits the study's predictive power. Indeed, selecting patients with resectable mCRC who are eligible for neoadjuvant chemotherapy appears to be challenging. However, to our knowledge, this is the first prospective study comparing the transcriptome of primary colon tumors and their matched liver metastatic lesions in resectable mCRC patients who were treated with neoadjuvant FOLFOXIRI. In addition, one of the strengths of this study was the systematic collection of two specimen sets, including colon tumor and liver metastasis, as well as normal colon and liver tissue before treatment, and the same data set after resection. Although we demonstrated a significant impact of FOLFOXIRI on gene expression in both primary tumor and liver metastases and identified *EPS8L2* as a potential biomarker of liver metastasis response, our data was preliminary; therefore, further studies with larger metastatic lesion cohorts are needed.

Conclusion

In summary, our mCRC case series demonstrated that the use of neoadjuvant FOLFOXIRI, both alone or in combination with cetuximab, leads to an alteration of the transcriptomic signature of primary colon tumors and matched liver metastatic loci. The pattern of DEGs changes between paired specimens of both primary tumor and metastases before and after treatment. Distinct patterns of DEGs are involved in the primary colon tumor and liver metastasis response. The EPS8L2 transcript identified in the liver metastatic lesion could serve as a candidate biomarker of liver complete response; however, prognostic conclusions cannot be drawn from this cohort.

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Disclosures

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Ethical approval: The study was approved by the Ethics Committee of the Tomsk Cancer Research Institute (Approval No. 2022–0122) and has been performed in accordance with the Helsinki Declaration and its later amendments.
Informed consent: Informed consent was obtained from all individual participants included in the study.
Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.
Author Contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by DE, AD, DK, SV, PG, SA, and NC**.** The first draft of the manuscript was written by NB and TD, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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