Tumor sidedness and enriched gene groups for efficacy of first-line cetuximab treatment in metastatic colorectal cancer

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Abstract

Molecular differences in tumor locations may contribute to the sidedness-specific response to cetuximab in metastatic colorectal cancer (mCRC). We investigated genes associated with the response to cetuximab treatment depending on tumor sidedness. Our study included 77 mCRC patients (13/63, right/left) with *KRAS* exon2 wild-type tumors from phase II trials of first-line therapy with cetuximab. Expression levels of 2551 genes were measured in tissue samples by *HTG EdgeSeq Oncology Biomarker Panel*. Univariate Cox regression analysis using log2 values of counts per million (CPM) was conducted in each sidedness to assess associations with clinical outcomes, and to define the optimal cutoff-point for clinically significant genes. In addition, a gene set enrichment analysis (GSEA) was performed to identify significant gene pathways in each sidedness. Sixty-nine patients were assessable for gene expression data. Overexpression of *BECN1* (log2(CPM) ≥ 6.8) was associated with favorable survival regardless of tumor sidedness. High-expression of *NOTCH1* (log2(CPM) ≥ 7.5) predicted significantly longer progression-free survival (median 14.7 vs. 11.1 months, HR 0.43, *P*=0.01) and overall survival (median 42.8 vs. 26.5 months, HR 0.35, *P*=0.01) in left side but not in right side. The GSEA showed that regulation of DNA replication gene set correlated with favorable survival in the left, while subcellular component and leukocyte migration gene sets were associated with good survival in the right. In conclusion, genes contributing to the efficacy of cetuximab treatment may differ according to the sidedness in mCRC. *NOTCH1* may potentially discriminate favorable responders to cetuximab in patients with left-sided tumors.

Introduction

The location of the primary tumor has an impact on clinical behavior and has prognostic value in metastatic colorectal cancer (mCRC). Patients with mCRC who harbor right-sided tumors have been shown to have poorer outcomes than those who harbor left-sided tumors. This phenomenon may derive in part from higher frequency of *BRAF* mutations, increased microsatellite instability and CpG island methylator phenotype, or higher incidences of mucinous differentiation and serrated pathway signature, which are more common in mCRC with right-sided primary tumors (1,2). In contrast, amplification of *EGFR* and *ERBB2*, chromosomal instability, and *TP53* gene mutations are more frequent in left-sided tumors (3). According to a sub-analysis of the CALGB80405 trial, primary tumor sidedness has been identified to be an independent prognostic marker in mCRC (4).

The molecular differences associated with sidedness in mCRC contribute in part to differences in the response to systemic treatment. Retrospective analyses of 2 first-line studies comparing chemotherapy plus cetuximab with chemotherapy plus bevacizumab reported better overall survival (OS) in the chemotherapy plus cetuximab group in patients with left-sided tumors. In contrast, patients with right-sided tumors appeared to receive more benefit from chemotherapy plus bevacizumab (5). Moreover, a recent meta-analysis suggested that tumor sidedness is a predictive marker of the response to anti-EGFR therapy in patients with RAS wild-type mCRC. Patients with left-sided tumors were shown to derive a greater benefit from chemotherapy plus anti-EGFR than from chemotherapy plus bevacizumab, while right-sided tumors were associated with trends toward detrimental effects of anti-EGFR therapy (6). On the basis of these results, the National Comprehensive Cancer Network (NCCN) guideline has recommended to consider the primary tumor site when

deciding the first-line treatment for mCRC (7). Actually, anti-EGFR therapy with cetuximab or panitumumab is recommended for only RAS wild-type and left-sided tumors. In addition, the pan-Asian adapted ESMO consensus guidelines have proposed that tumor sidedness matters when we treat patients with RAS/*BRAF* wild-type tumors (8).

Although increasing evidence suggests that tumor sidedness is a predictor of the response to anti-EGFR antibodies, this does not mean that all patients with right-sided tumors should clinically avoid receiving anti-EGFR antibodies as an initial treatment. Data from prospective clinical trials have demonstrated that a few patients with right-sided tumors had a good depth of response and that even though a durable response was achieved in a larger proportion of patients with left-sided tumors than in patients with right-sided tumors, a few patients with right-sided tumors also had rapid and deep responses (9). Some patients with right-sided tumors may be responders who benefit from anti-EGFR antibodies and should receive anti-EGFR-based chemotherapy as an initial treatment. Therefore, there may be biomarkers to elucidate the subset of mCRC patients who are likely to benefit from anti-EGFR treatment in each side.

We therefore performed a biomarker study to establish responders to anti-EGFR treatment using tissue samples obtained from patients enrolled in prospective clinical trials. The aim of this study was to investigate which genes are associated with the response to cetuximab treatment depending on tumor sidedness in patients with mCRC who received first-line cetuximab treatment.

Materials and Methods

Study design and patient population

We retrospectively collected tissue samples from 2 prospective clinical trials which evaluated combination therapy with cetuximab and oxaliplatin-based chemotherapy as first-line treatment, the modified-FOLFOX6 regimen (JACCRO CC-05: N=57, UMIN000004197) (10) and the SOX regimen (JACCRO CC-06: N=67, UMIN000007022) (11) for mCRC patients with *KRAS* wild-type tumors (**Supplementary Figure 1**). This biomarker study was conducted in accordance with the Declaration of Helsinki and was approved by the ethical committee of each participating institute. Written informed consent was obtained from the patients before enrollment. If the investigators could not obtain informed consent, the patient was eligible for enrollment under permission by the institutional review board of each institute. Tissue samples at the time of biopsy or surgery before chemotherapy were collected.

Assessment of efficacy

The endpoints of this biomarker study were progression-free survival (PFS) and OS. The JACCRO trials included the same secondary endpoints of OS and PFS based on disease progression detected by external review or death from any cause. Disease progression was evaluated according to RECIST, version 1.1 by the investigators and was then validated by an external review board.

RNA isolation and gene expression analysis

Formalin-fixed, paraffin-embedded (FFPE) tumor specimens from primary tumor site were cut into sections with a thickness of 3 or 10 μm. A pathologist stained one 3-μm slide with hematoxylin and eosin and then evaluated for tumor content and marked for areas with

dominant tumor foci for the preparation of macrodissection. Macrodissection was performed by scraping the marked areas with a blade to ensure that as many tumor cells as possible were dissected. Total RNA was extracted from formalin-fixed paraffin-embedded tissue of the tumor samples using an miRNeasy FFPE Kit (QIAGEN KK, Tokyo, Japan) according to the manufacturer's protocol.

Gene expression levels were measured by the *HTG EdgeSeq Oncology Biomarker Panel*, with probes targeting 2551 genes implicated in numbers of pathways, using next generation sequencing for quantitative analysis of targeted genes (https://www.htgmolecular.com/assays/obp).

This study was conducted in accordance with the REporting recommendations for tumor MARKer prognostic studies (REMARK) (12). Tissue analyses were performed blindly to the clinical dataset at HTG Molecular, Inc. (AZ, United States) after approval in the Institutional Review Board of each institution which participated in the JACCRO CC-05/06AR trials (UMIN000010635).

Statistical evaluation

The R statistical software (version 3.3.2, https://www.r-project.org/) for the survival analyses of the gene expression profiles. Univariate Cox regression analysis using log2 values of counts per million (CPM) was conducted for all genes that passed QC filtering in each side to assess the association with clinical outcomes. A *P*-value of less than 0.05 was considered as statistically significant. Further univariate Cox regression analysis was performed to define an optimal cutoff point for significant genes. In addition, a gene set enrichment analysis (GSEA, http://software.broadinstitute.org/gsea) was performed to identify

classes of genes associated with outcomes in each side by the GSEA Preranked analysis based on the hazard ratio of each gene calculated during the univariate Cox regression analysis. The biological process entries in Gene Ontology terms (category c5.bp) was used for the target gene sets for GSEA. The gene sets satisfying both $P < 0.05$ and false discovery rate (FDR) < 0.25 were considered statistically significant.

SAS 9.0.3 software (SAS Institute, Cary, NC, USA) was used to perform all analyses unless specified otherwise. All tests were 2-sided with a significance level of 0.05.

Results

Patient characteristics

A total of 77 patients were studied. Sixty-three (82%) of the patients had left-sided tumors, and 13 (17%) had right-sided tumors. The patient characteristics are summarized in **Supplementary Table 1**. There were no statistically significant differences in characteristics between patients with left-sided tumors and those with right-sided tumors. In the enrolled patients, the ORR was 73%. The median PFS and OS were 10.0 months (95% CI 8.8–11.8 months) and 33.9 months (95% CI 26.5–not reached), respectively. Univariate Cox regression analysis included 69 patient samples that passed the internal Quality Control metrics of HTG EdgeSeq Oncology Biomarker Panel.

Identified significant genes in both sides

Overexpression of *BECN1* (log2(CPM) ≥ 6.8) was associated with favorable OS for both left-sided tumors and right-sided tumors. On the other hand, there was no gene which correlated with bad OS in both left-sided tumors and right-sided tumors (**Figure 1**). In the

analysis of PFS, no gene was significantly associated with PFS in both left-sided tumors and right-sided tumors (**Figure 2**).

Promising significant genes to predict prognosis in each side

In the left-sided tumor group (n=60), the Cox regression analysis identified 16 genes associated with clinical outcomes for PFS and 26 genes associated with clinical outcomes for OS (P<0.01). Six of the 16 genes associated with PFS and 18 of the 26 genes associated OS were associated with favorable survival. *BECN1* and *NOTCH1* genes were identified to be positively associated with both better PFS and better OS. When the cutoff point was defined as log2(CPM) 7.5, patients with *NOTCH1* high-expression had significantly longer PFS (median 14.7 months vs. 11.1 months, HR 0.43, 95% CI 0.22-0.81, P=0.01) and OS (median 42.8 months vs. 26.5 months, HR 0.35, 95% CI 0.15-0.79, P=0.01) compared to those with *NOTCH1* low-expression in the left-sided tumor group, but not in the right-sided tumor group (**Figure 3**).

In the right-sided tumor group (n=9), the Cox regression analysis showed that 44 genes were associated with clinical outcomes for PFS, and 33 genes were associated with clinical outcomes for OS (P<0.01). Eleven of the 44 genes associated with PFS and 2 of the 33 genes associated with OS were associated with favorable survival. Moreover, the analysis identified 4 genes (*CST6*, *FGF18*, *SHC3*, and *TMEM57*) that were associated with both worse PFS and worse OS. There was no gene significantly associated with both better PFS and OS.

GSEA identified significant pathways associated with outcomes of tumors on each side

In the GSEA, we identified gene sets of left-sided tumors and right-sided tumors that

were associated with PFS or OS and had *P*-values of less than 5% and false discovery rates of less than 25% (**Table 1 and Table 2**).

In the left-sided tumor group, one gene set regarding regulation of DNA replication was significantly associated with better OS. Sixteen gene sets correlated with better PFS, while 4 gene sets were associated with worse PFS. Among 16 gene sets, 3 gene sets related to angiogenesis, extracellular structure, or chromatin organization were strongly associated with favorable PFS (FDR<0.05).

In the right-sided tumor group, 13 gene sets correlated with better PFS. Ten gene sets were associated with better OS, while 10 other gene sets correlated with worse OS. In particular, gene sets of organonitrogen compound biosynthetic process and translational initiation were strongly associated with favorable PFS. Subcellular component and leukocyte migration genes correlated with better OS, whereas gene sets of DNA metabolic process, DNA repair, and cellular response to DNA damage stimulus were strongly associated with worse OS (FDR<0.05) (**Figure 4**).

Discussion

Our study demonstrated that there were significant differences in gene expression levels which were associated with clinical outcomes between primary tumor sidedness in mCRC patients treated with first-line cetuximab-based chemotherapy. A pooled analysis of 6 randomized trials evaluating the prognostic and predictive values of primary tumor sidedness in patients with RAS wild-type mCRC confirmed that adding an anti-EGFR drug had a greater effect than adding bevacizumab; this effect was greatest in patients with left-sided tumors (6). However, it has been reported that some patients with right-sided tumors respond to

chemotherapy combined with anti-EGFR antibody (9,13). In present study, we found 16 genes in the left-sided tumor group and 44 significant genes in the right-sided tumor group that were significantly associated with PFS. There were no common genes that were significantly associated with PFS in both tumor side groups. Moreover, in the GSEA, gene sets associated with PFS differed between the left-sided and right-sided tumor groups. This finding suggests that gene expression signatures may explain differences in cetuximab efficacy dependent on tumor sidedness.

Patients with left-sided tumors are more likely to respond to anti-EGFR antibodies, leading to survival benefit. However, some patients do not respond to anti-EGFR therapy even if they have left-sided primary tumors, indicating that RAS and sidedness may be not a sufficient predictor of the response to anti-EGFR antibodies. There is an urgent need for predictive biomarkers for anti-EGFR therapy to identify certain responders among mCRC patients with left-sided primary tumors. Our findings suggest that *NOTCH1* gene expression is significantly associated with survival in the left-sided tumor group, but not in the right-sided group. A family of membrane-bound receptors related to the NOTCH proteins (NOTCH1, NOTCH2, NOTCH3, and NOTCH4) contribute to regulating tumor cell proliferation, differentiation, apoptosis, and migration (14,15). Several biomarker studies have reported that increased *NOTCH1* expression is associated with lymph node metastasis in CRC (16). Moreover, *NOTCH1* high-expression had multivariable associations with poor outcomes in CRC (16-19). NOTCH1 mediates the resistant effects of regorafenib in colorectal cancer cells (20). It has also been shown that *NOTCH1* expression is a detrimental prognostic factor in mCRC patients who receive chemotherapy plus the anti-VEGF antibody bevacizumab (21). *NOTCH1* expression may contribute to tumor resistance to bevacizumab by inducing

angiogenesis, which generates large vessels that increase the tumor blood supply and diminish the sensitivity to bevacizumab (22). In our study, high expression of the *NOTCH1* gene was associated with favorable clinical outcomes in patients with left-sided tumors who received cetuximab-based chemotherapy. This finding indicates that patients with mCRC harboring *NOTCH1*-high tumors may receive more benefit from cetuximab and that the gene expression might be an useful marker for identifying patients who are likely to benefit from anti-EGFR antibody or bevacizumab as first-line treatment for RAS wild-type mCRC.

The GSEA indicated that gene sets associated with cell cycle phase transition and nucleoside monophosphate biosynthesis were related to poor PFS in patients with left-sided tumors who received cetuximab plus chemotherapy. The cell phase transition is the cell cycle process by which a cell commits to entering the next phase of the cell cycle. Cell cycle progression is related to cancer cell proliferative activity, while cell cycle arrest promotes apoptosis and autophagy of colon tumor cells. Epithelial growth factor (EGF), via its receptor (EGF receptor [EGFR]), elicits proliferation in many human cancers (23). Cetuximab occludes the binding sites of EGFR, and EGFR signaling is one of the main drivers of colon cancer growth. Previous studies have reported that cetuximab obstructs the cell cycle in G1 phase (24). Conversely, the impairment of the EGF/EGFR system induced by cetuximab might be reduced by activation of the cell cycle transit phase pathway. Additionally, in our study, the most significant gene contributing to poor outcomes in the left-sided tumor group was the *ALDH1A1* gene (P=0.0022). It has also been reported in CRC that aldehyde dehydrogenase 1A1 (ALDH1A1) is an immunohistological biomarker of various solid tumors (25). Several studies have suggested that ALDH1A1 may be a biomarker of cancer stem cells and can be used as a prognostic predictor of CRC (26). A biomarker study of CRC indicated that the

ALDH1A1 expression was not related to differences in survival time (27); however, it has been shown that nuclear expression of *ALDH1A1* is significantly associated with shorter OS and that the ratio of the *ALDH1A1* level in adjacent mucosa to that in tumor tissue is closely related to invasion, metastasis, and prognosis in CRC (25,28). A previous molecular subtyping study demonstrated that a subtype that is stem-like and includes upregulation of genes involved in matrix remodeling and epithelial-mesenchymal transition carries a very poor prognosis and, moreover, is refractory to EGFR-targeted therapy (29). Therefore, the *ALDH1A1* gene may be a prognostic factor but may be also a predictor of a poor response to cetuximab in mCRC.

In the right-sided tumor group, the *CST6*, *FGF18*, *SHC3*, and *TMEM57* genes were associated with worse PFS and OS. Cystatin 6 (CST6) has been considered to be a tumor-suppressor in breast tissue (30), reducing breast cancer cell proliferation, adhesion to endothelial cells, matrigel invasion, and migration (31), but this has not been reported in CRC. Loss of *CST6* gene expression has ascribed promoting hypermethylation in breast cancer (30). Cystatin M, a protein coded by the *CST6* gene, which controls the activity of legumain, is found to be a oncogene and an indicator of a poor prognosis in colorectal and breast cancers, but also to be overexpressed in the majority of human solid tumors (32). Fibroblast growth factor receptor 3 (FGFR3) has been reported to negatively regulate bone growth (33) and also to be involved in carcinogenesis. Up-regulation of fibroblast growth factor 18 (FGF18), one of ligands of FGFR3, was shown to have oncogenic impact (34) and to lead malignant cell growth and survival in human CRC cell lines (35). The FGF-receptor splice variant FGFR3-IIIc mediates FGF18-dependent signaling. In colon adenoma cells, an FGF18/FGFR3-IIIc autocrine growth and survival loop is up-regulated in a Wnt-dependent manner and controls

tumor cell growth (36). Several types of genes associated with tumor growth may contribute to poorer outcomes in mCRC with right-sided primary tumors.

The *BECN1* high-expression was found to be significantly associated with favorable survival in both tumor sidedness groups of our study. BECN1 has an important role in canonical autophagy, to regulate autophagic phosphatidylinositol 3-phosphate generation and recruit additional ATG proteins for autophagosome formation (37). Autophagy-related genes are over-regulated or down-regulated in cancers, but also significantly correlate with poor prognoses, suggesting the complex biological role of autophagy in cancer (38,39). Monoallelic loss of the *BECN1* gene causes susceptibility to metabolic stress and promotes tumorigenesis (40). A retrospective review from clinicopathological and immunohistochemical data indicated that the absence of autophagy-related protein expression correlated with poor prognosis in CRC; therefore, suggested that these proteins may be novel prognostic markers (41). Our results suggest that *BECN1* may be a promising gene for predicting favorable outcomes in mCRC.

Our study had several limitations that must be taken into account when interpreting the results. A major limitation was that our study population may include patients with non-exon 2 *KRAS* and *NRAS* mutations. An extended RAS test is now recommended for patient selection for anti-EGFR therapy as it is known that patients with non-exon 2 *KRAS* and *NRAS* mutations do not derive benefit from anti-EGFR antibodies (42,43). These patients should be excluded from the analysis; however, it was not able to check RAS status due to lack of the remaining tissues samples. The sample size of patients with right-sided primary tumors (n=13) was very small in this study; therefore, it may be not statistically reliable. It is difficult to exactly assess the impact of candidate genes on the effectiveness of cetuximab since the

patient cohort comprised only patients who received cetuximab, meaning that we are unable to evaluate the genes as predictive markers. We found that there were no common genes and pathways that were significantly associated with PFS in both the left-sided and right-sided tumor groups; however, this may have resulted from the small study group. To resolve these limitations, our findings will be validated using data of an on-going randomized trial, the DEEPER (NCT02515734), which evaluates triplet-regimen plus cetuximab or bevacizumab as first-line treatment for RAS wild-type mCRC.

In conclusion, our data suggest that genes contributing to the response or resistance to cetuximab treatment may differ between right-sided tumors and left-sided tumors in patients with mCRC. *NOTCH1* may potentially discriminate certain responders to cetuximab in patients with left-sided primary tumors, while several genes may contribute resistance to cetuximab in patients with right-sided primary tumors. These findings need to be confirmed in studies using larger cohorts with RAS wild-type mCRC patients.

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Table 1. Results of gene set enrichment analysis for progression-free survival

	Left			Right		
		NES	FDR		NES	FDR
Good prognosis	VASCULATURE DEVELOPMENT	-2.644	0.022	ORGANONITROGEN COMPOUND BIOSYNTHETIC PROCESS	-2.914	0.002
	EXTRACELLULAR STRUCTURE ORGANIZATION	-2.617	0.022	TRANSLATIONAL INITIATION	-2.713	0.011
	BLOOD VESSEL MORPHOGENESIS	-2.664	0.028	LEUKOCYTE MIGRATION	-2.419	0.054
	CHROMATIN MODIFICATION	-2.735	0.029	AMIDE BIOSYNTHETIC PROCESS	-2.421	0.067
	CHROMATIN ORGANIZATION	-2.489	0.046	PEPTIDE METABOLIC PROCESS	-2.444	0.075
	ANGIOGENESIS	-2.451	0.050	CELLULAR AMIDE METABOLIC PROCESS	-2.328	0.083
	REGULATION OF VASCULATURE DEVELOPMENT	-2.386	0.066	DICARBOXYLIC ACID METABOLIC PROCESS	-2.224	0.115
	SKELETAL MUSCLE ORGAN DEVELOPMENT	-2.342	0.079	ACUTE INFLAMMATORY RESPONSE	-2.211	0.115
	MULTICELLULAR ORGANISM METABOLIC PROCESS	-2.297	0.097	SMALL MOLECULE METABOLIC PROCESS	-2.229	0.125
	MULTICELLULAR ORGANISMAL MACROMOLECULE METABOLIC PROCESS	-2.193	0.146	ORGANONITROGEN COMPOUND METABOLIC PROCESS	-2.235	0.137
	REGULATION OF BLOOD CIRCULATION	-2.193	0.159	RESPONSE TO PROSTAGLANDIN	-2.132	0.158
	CHROMATIN REMODELING	-2.203	0.165	G PROTEIN COUPLED RECEPTOR SIGNALING PATHWAY	-2.114	0.164
	REGULATION OF CELLULAR COMPONENT MOVEMENT	-2.137	0.170	CELL CHEMOTAXIS	-2.136	0.169
	CIRCULATORY SYSTEM DEVELOPMENT	-2.120	0.177			
	REGULATION OF GENE EXPRESSION EPIGENETIC	-2.111	0.177			
	INFLAMMATORY RESPONSE	-2.139	0.179			
Bad prognosis	CELL CYCLE PHASE TRANSITION	2.725	0.026			
	NUCLEOSIDE MONOPHOSPHATE BIOSYNTHETIC PROCESS	2.500	0.068			
	CELL CYCLE G1 S PHASE TRANSITION	2.242	0.201			
	PROTEIN DEPHOSPHORYLATION	2.269	0.225			

Abbreviations: NES, normalized enrichment score; FDR, false discovery rate.

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Table 2. Results of gene set enrichment analysis for overall survival

	Left			Right		
		NES	FDR		NES	FDR
Good prognosis	REGULATION OF DNA REPLICATION	-2.265	0.235	MOVEMENT OF CELL OR SUBCELLULAR COMPONENT	-2.722	0.034
				LEUKOCYTE MIGRATION	-2.603	0.042
				SMALL MOLECULE METABOLIC PROCESS	-2.402	0.060
				TAXIS	-2.352	0.064
				CELL CHEMOTAXIS	-2.403	0.071
				REGULATION OF PROTEIN MATURATION	-2.354	0.072
				POSITIVE REGULATION OF TRANSCRIPTION FROM RNA POLYMERASE II PROMOTER IN RESPONSE TO STRESS	-2.295	0.078
				ION HOMEOSTASIS	-2.302	0.083
				CELL MOTILITY	-2.409	0.086
				RESPONSE TO HORMONE	-2.230	0.091
Bad prognosis				DNA METABOLIC PROCESS	2.694	0.015
				DNA REPAIR	2.700	0.030
				CELLULAR RESPONSE TO DNA DAMAGE STIMULUS	2.472	0.047
				MITOTIC CELL CYCLE	2.489	0.055
				CELL CYCLE PROCESS	2.339	0.066
				NEGATIVE REGULATION OF CHROMOSOME SEGREGATION	2.348	0.069
				PROTEIN MODIFICATION BY SMALL PROTEIN CONJUGATION OR REMOVAL	2.353	0.077
				NEGATIVE REGULATION OF CELLULAR PROTEIN CATABOLIC PROCESS	2.369	0.083
				PROTEIN PHOSPHORYLATION	2.280	0.084
				PEPTIDYL AMINO ACID MODIFICATION	2.248	0.092

Abbreviations: NES, normalized enrichment score; FDR, false discovery rate.

Figure legends

Figure 1. Identified significant genes associated with overall survival by tumor sidedness

Figure 2. Identified significant genes associated with progression-free survival by tumor sidedness

Figure 3. Kaplan-Meier curves of overall survival and progression-free survival by *NOTCH1* expression level in patients with left-sided primary tumors

Figure 4. Enriched genes and gene pathways for outcome of cetuximab treatment in each side

Figure 1

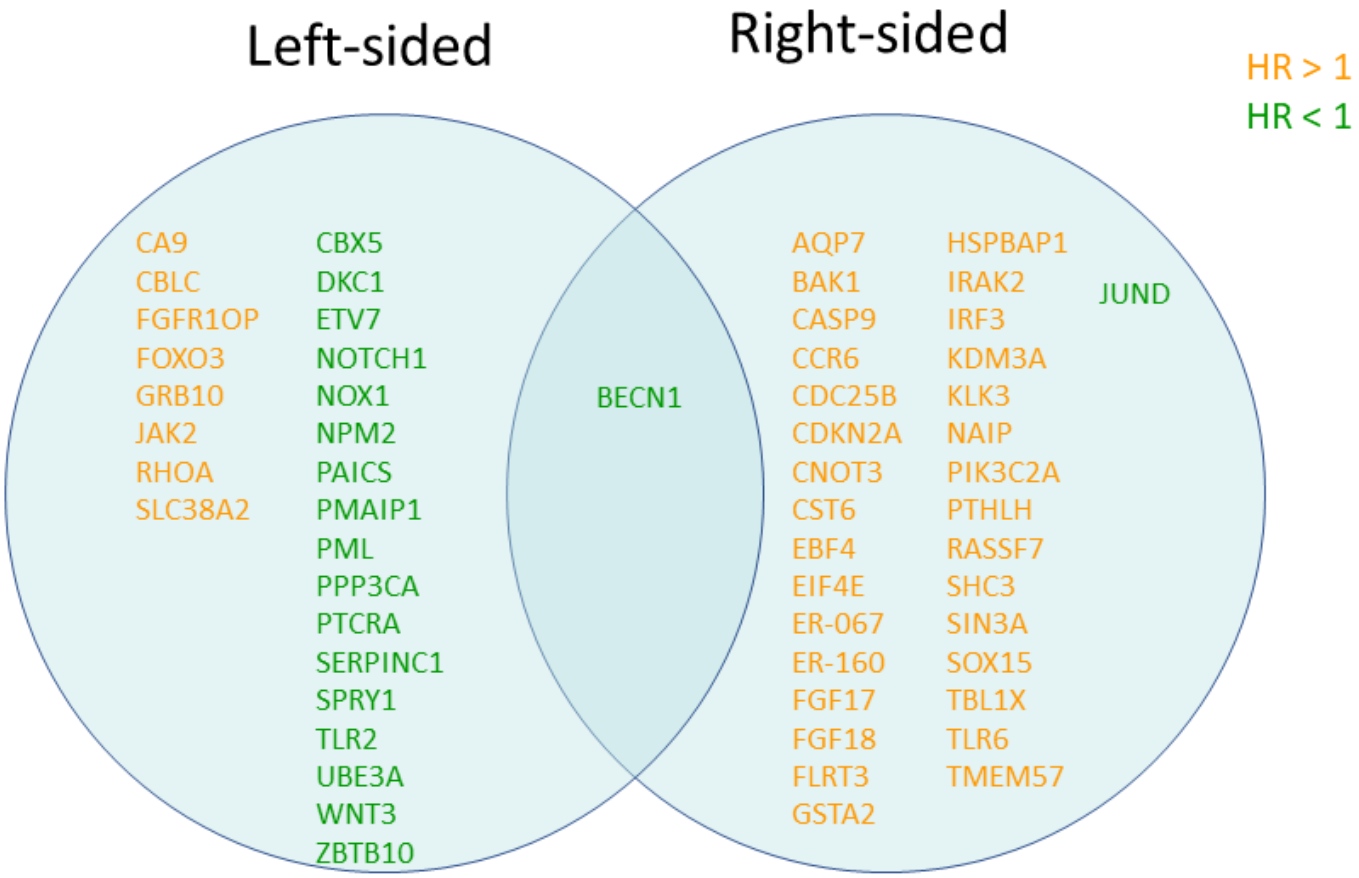


Figure 2.

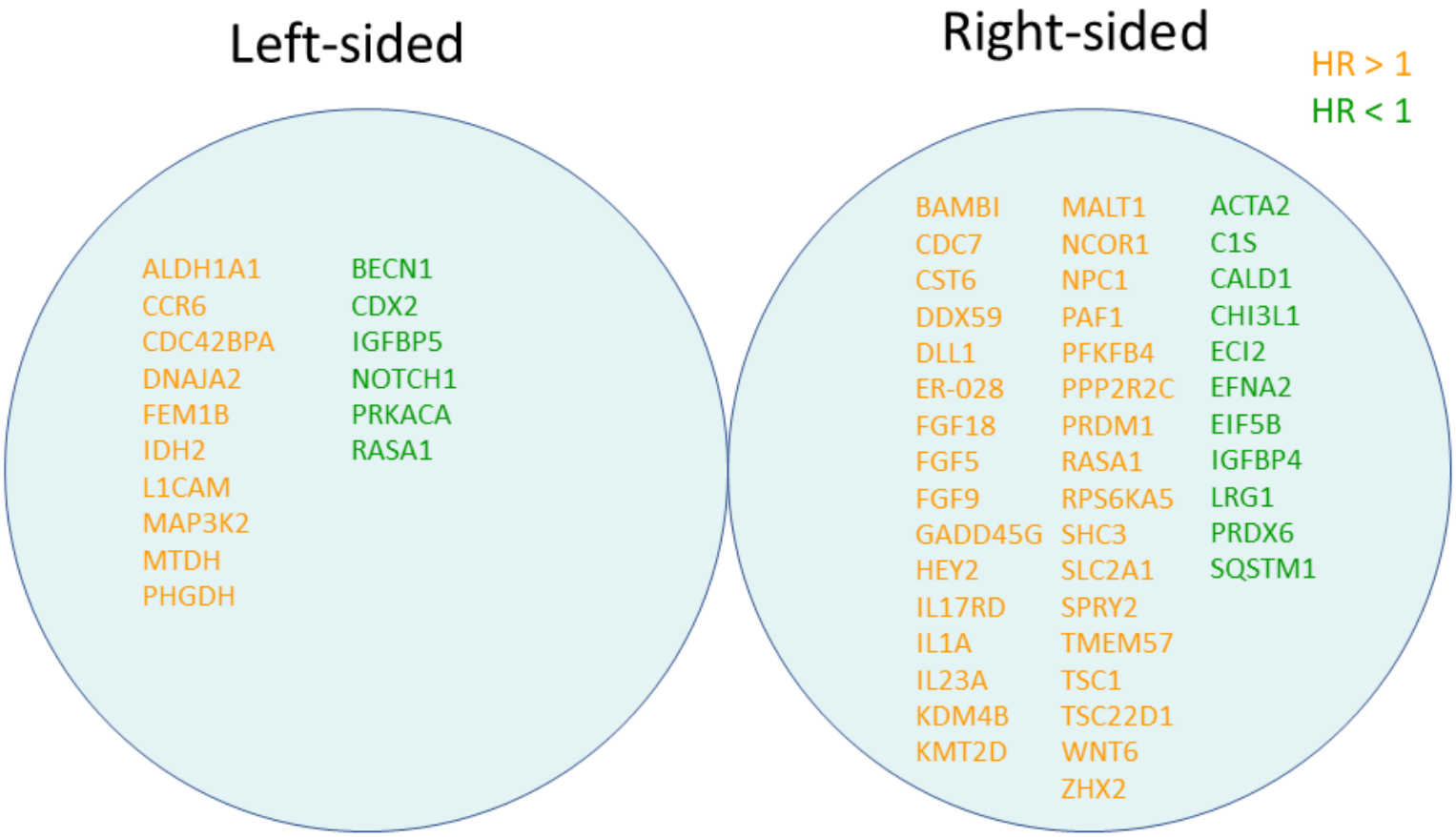


Figure 3

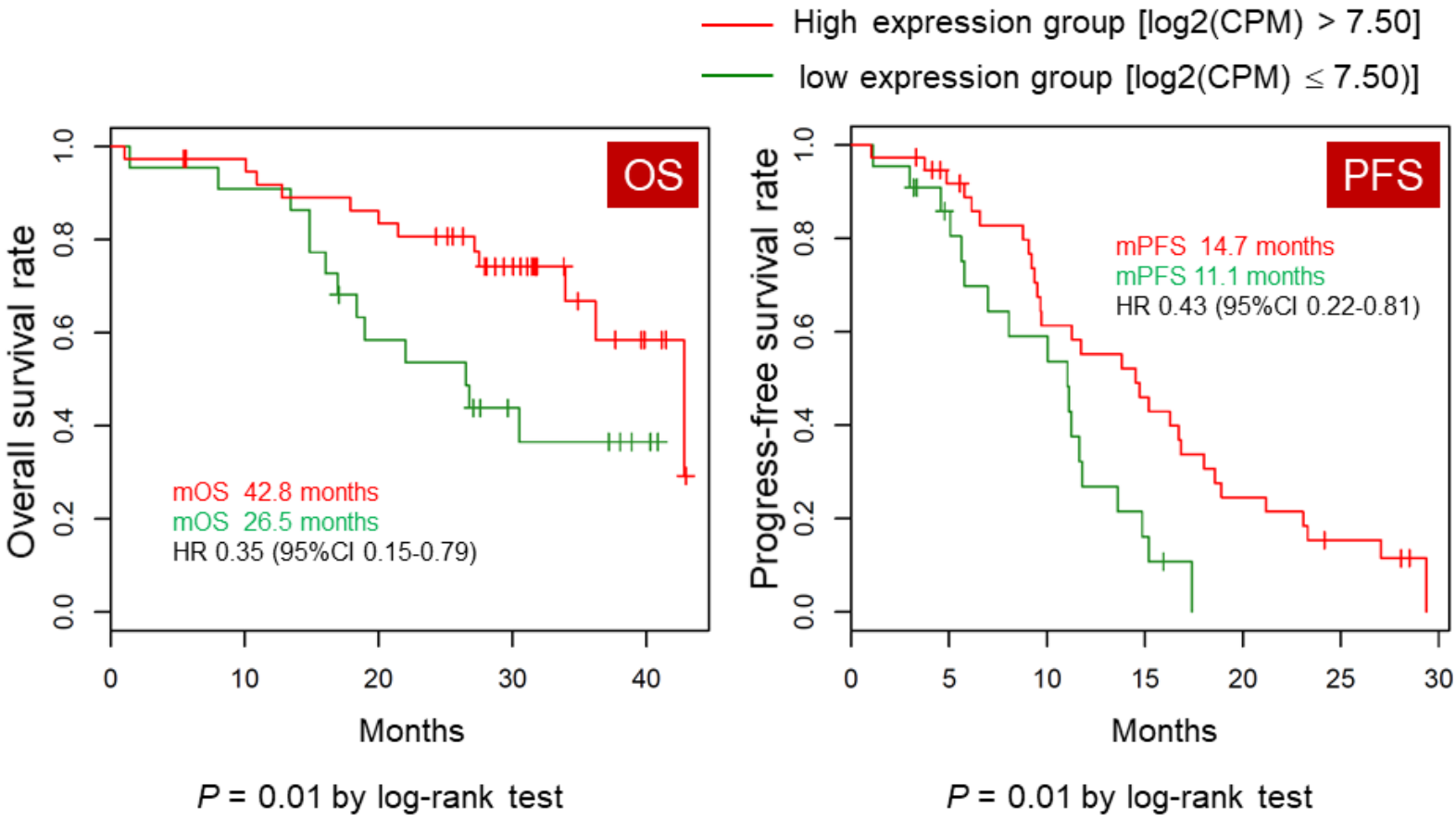
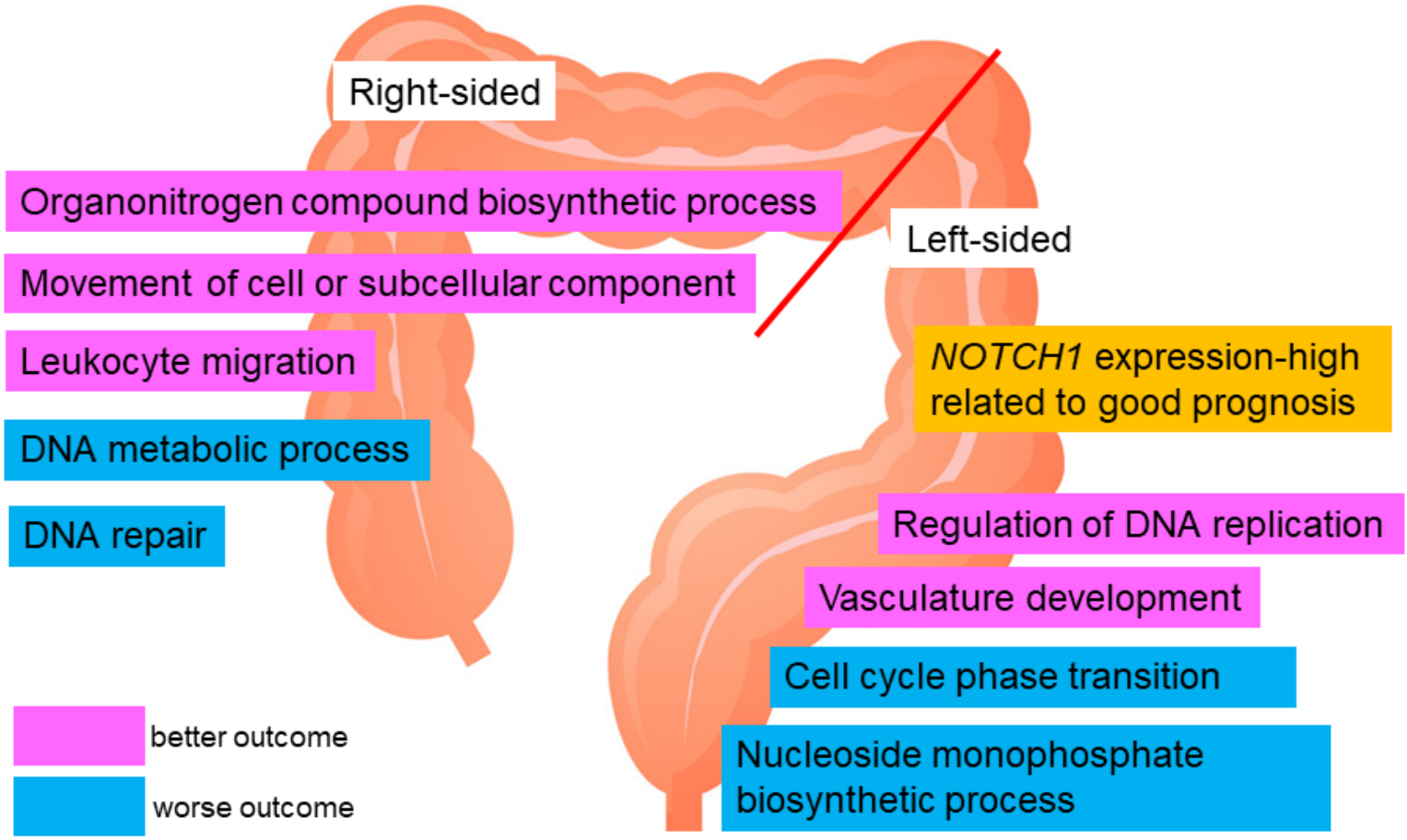


Figure 4



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