

## Original Article

# JAK2-dependent proliferation of Fas positive colorectal cancer cells may associate with poor prognosis in colorectal cancers

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Received January 19, 2017; Accepted February 22, 2017; Epub May 1, 2017; Published May 15, 2017

**Abstract:** JAK2 is considered as an important cytokine mediator in some malignant tumors which can promote tumor growth. Fas is well known as pro-apoptotic receptor on cell membrane which can modulate cell immunity and apoptosis. However, recent studies show that Fas is emerging as a positive modulator in colorectal cancer (CRC) progression. The mystery of Fas makes it worth to explore the way to drive Fas to pro-apoptotic property. The purpose of this research is to investigate the role of JAK2 in Fas (+) CRC cells. We studied the correlation between Fas expression, JAK2 expression and survival in separate human colorectal cancer (CRC) cohorts by Kaplan-Meier survival curve and analysis of gene expression profiles. Fas expression in non-metastasis CRC is associated with poor prognosis. Fas is co-expressed with genes involved in chemokine signaling pathway, antigen processing and presentation, cytokine-cytokine receptor interaction and apoptosis, among which the top is JAK2. IHC on paraffin-embedded CRC tissue sections and western blots for CRC patient-originated cells were probed using Fas and JAK2 antibody, revealing that Fas and JAK2 are co-expressed in CRC tumor tissues as well as in cells. Scoring for IHC and quantification for western blots also link JAK2 to Fas ( $P < 0.05$ ). In vitro clone formation assays reveal that JAK2 inhibition markedly suppress clone formation capacity in majority of CRC cell lines. JAK2 inhibition alone doesn't seem to induce apoptosis, and doesn't seem to cooperate with FasL in apoptosis. These results indicate that JAK2 may serve as a biomarker in predicting prognosis for CRCs. This paper also shows JAK2 may positively regulate CRC stem-like cell proliferation, which emerges to be a novel therapeutic target for Fas (+) CRCs.

**Keywords:** JAK2, Fas, colorectal cancer, prognosis, mechanism

## Introduction

Recently, JAK2 is considered as a promising target in treating many malignant tumors [1]. JAK2 is a critical molecule in that phosphorylated JAK2 (pJAK2) in activated form can transmit signals via JAK/STAT pathway from outside the cells to the inside nucleus involving differentiation and cell growth. Given that phosphorylation of JAK2 depends on the presence and binding of some immune molecules on cell membranes, we presumed that those colorectal cancer cells expressing Fas on the membrane may recruit more immune cells with the consequence of more immune molecules produced in tumor microenvironment.

Death receptors have been implicated in apoptosis in multiple cell types. Among these death

receptors is FasL/Apo-1. Stimulation of Fas activates several cellular signaling pathways. The biofunctions of Fas are regulated through at least five levels, including extracellular signaling, membrane signal transduction (the assembly of DISC), DISC, mitochondrial level, and miRNA. As a consequence, the result of cell fate is either pro- or antiapoptosis [2].

Some studies have shown that many cancer cell lines expressed large quantities of Fas, and were highly sensitive to Fas ligand (FasL) induced apoptosis [3]. The pro-apoptotic properties of Fas transmembrane signaling have been extensively studied over the last few decades. Under immunological surveillance, cancer cells are eliminated through Fas induced apoptosis. Fas receptors on cancer cell membrane are recognized by and bind to FasL which

is required for Fas stimulation and followed by clustering of the membrane receptors and the recruitment of FADD (Fas-associated protein with death domain) [4]. FADD and subsequent caspase-8 activation, together with Fas and FLIP (FLICE-like inhibitory protein), form the death-inducing signaling complex (DISC) [5, 6], which is required for apoptosis [7]. However, Fas receptors not only play an apoptotic role on stimulation but also bring immunological responses to dying cells and their microenvironment [8]. During the process of apoptosis induced by Fas, cells can secrete several cytokines such as IL-6, IL-8, MCP-1, which can recruit phagocytes and subsequent engulfment to dying cells. Nevertheless, Fas is now emerging as an important molecular in promoting cell proliferation [9] and/or migration [10-14]. Animal experiments found that loss of Fas reduced cancer incidence as well as tumor burden [15], suggesting that Fas may play a positive role in tumorigenesis. Some in vitro studies report that Fas is involved in cell proliferation and survival through activation of some other major tyrosine signaling pathways. Considering that among the pathogenesis of colorectal tumors, inflammatory bowel diseases (IBD) and colorectal polyps are major risks where Fas is largely involved [13], and that Fas can act as either a pro-apoptotic or a proliferation mediator, it is worth to know how colorectal cancer cells respond to Fas stimulation, so as to further try to make apoptosis instead of proliferation potential through Fas.

In this paper, we show that Fas expression in human colorectal cancers (CRC) is strongly correlated with JAK2 expression, patients' survival, especially in non-metastatic colorectal cancers. We also found that JAK2 plays an important role in Fas associated gene networks. JAK2 inhibition can suppress tumor colony formation in cell cultures by using JAK2 specific inhibitors working in medium/Matrigel culturing system.

### Materials and methods

#### *Bioinformatics study*

Clinical data were collected from R2 microarray analysis and visualization platform (R2) and colorectal patients from UMC. Expression level of Fas and JAK2 were investigated in all colorectal cancer cohorts through the Mega sampler function in R2 across Datasets.

In R2 Single Dataset function, overall survival (OS) data were available in two datasets (Smith et al [16], Sieber et al [17]). Disease-free survival (DFS) data were available in the above dataset. The association of JAK2 or FAS with DFS and OS was determined by using Kaplan Meier curve from R2 Single Dataset function. Median Fas or JAK2 expression levels were used as cutoff values. *P* values were determined by log-rank test as described in Bewick et al [18].

Fas associated pathways were analyzed first by choosing the "KEGG Pathway Finder" option and setting the gene correlation (Pearson correlation) *P*-values < 0.01 for each dataset, providing that Pearson's correlation coefficient follows t-distribution with *n*-2 degrees of freedom. The Fas associated pathways that were significantly enriched in at least two datasets (chi-square test with continuity correction is performed; *P*-value < 0.01) were identified and genes were then ordered according to the significance, which was determined based on the combined *P* values (Stouffer z-trend) that were calculated using the web Meta P application (<http://compute1.lsrc.duke.edu/software/MetaP/metap.php>). Then Gene Ontology (GO) analysis was done via similar approach to get significantly enriched lists of Fas-associated pathways and genes. The lists of genes were generated then for each dataset.

Using the STRING analysis web-based application ([www.string-db.org](http://www.string-db.org)), the functional interconnectivity between the genes on the lists was shown in form of network so that the protein interactions were visualized. The subsets of genes especially the communal subset in significant association with Fas were identified via "GeneVenn" web application (<http://mcbbc.usm.edu/genevenn/>) among three data sets.

The heat maps were generated by reimporting those genes into R2 database. Here, the genes were from the largest dataset available (Smith et al, 232 tumors) and the tumors were ordered according to Fas expression levels from low to high.

All patients' materials were collected after approval by patients and in accordance with related rules and guidelines of Medical Ethical Committee of the University Medical Center Utrecht (UMCU).

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### *Cell lines and cultures*

Human originated colorectal cancer cell lines CRC29, CRC47, CR9, CR16, CRC48, CRC26<sup>47</sup>, and liver metastatic cell lines L169, L167, L145 were cultured in DMEM/F12 (Gibco) supplemented with DMEM/F12 supplemented with 0.5% glucose (Sigma), 1% Ultraglutamine 1 (Lonza), 0.5% Trace element A (Cellgro), 1% Trace element B (Cellgro), 1% Trace element C (Cellgro), 5 mM HEPES Buffer (Lonza), 100 µg/ml Apo transferrin (Sigma), 20 µg/ml Insulin (Sigma), 5.6 ng/ml Progesterone (Sigma), 4.7 ng/ml Sodium Selenite (Sigma), 1 µg/ml GSH (Sigma), 8.6 µg/ml Putrescine (Sigma), 100 µM β-mercaptoethanol (Merck), 10 ml of antibiotic-antimycotic (Gibco), 4 µg/ml gentamicin (Invitrogen), 0.2% lipid mixture (Sigma).

### *Antibodies, inhibitors and other reagents*

The following antibodies were purchased from, Inc, Leiden, The Netherlands: rabbit JAK2 (#3230), rabbit p-STAT3 (#9131), mouse STAT3 (#9139s). Fas antibody rabbit Fas C-20 (sc-715) was obtained from Santa Cruz Biotechnology, Inc, Heidelberg, Germany. Secondary peroxidase-conjugated antibodies were goat anti-mouse-HRP or goat anti-rabbit-HRP obtained from Dako (Glostrup, Denmark). JAK2 inhibitor CEP33779 were provided by Sellechem Incorporation. Dimethyl sulfoxide (DMSO) was ordered from Sigma Inc.

### *Immunohistochemical staining*

After excision, samples of primary colorectal cancer or liver metastasis were fixed in 4% paraformaldehyde and paraffin embedded. Paraffin-embedded tissues were sectioned by Leica microtome RM 2235 and attached to glass slides. Immunohistochemistry for FAS and JAK2 were stained and scored. Staining was carried out according to standard immunohistology protocol from cell signaling. Briefly speaking, deparaffinize and rehydrate the slides in xylene, 100% alcohol, 95% alcohol and 80% alcohol in order followed by heat-mediated antigen retrieval in pH 6.0 citrate buffer. Incubate in 3% peroxide block buffer at room temperature for 15 minutes and PBS pH 7.4 wash for three times, 5 min for each. Add FAS antibody (1:500) and JAK2 antibody (1:1000) respectively, and incubate at 4°C overnight. Never let the slides dry during the staining.

Wash the slides with PBS-Tween for three times, 5 min for each. Incubate with horseradish peroxidase-labeled Bright Vision secondary antibody at room temperature for 1 hour. Detect with DAB and re-stain with HE. Wash, dehydrate and mount the slides. Image acquisition was performed on Nikon Eclipse E800M photomicroscope connected with a computer. A semi-quantitative scoring criterion for IHC was used, in which both staining intensity and positive areas were recorded.

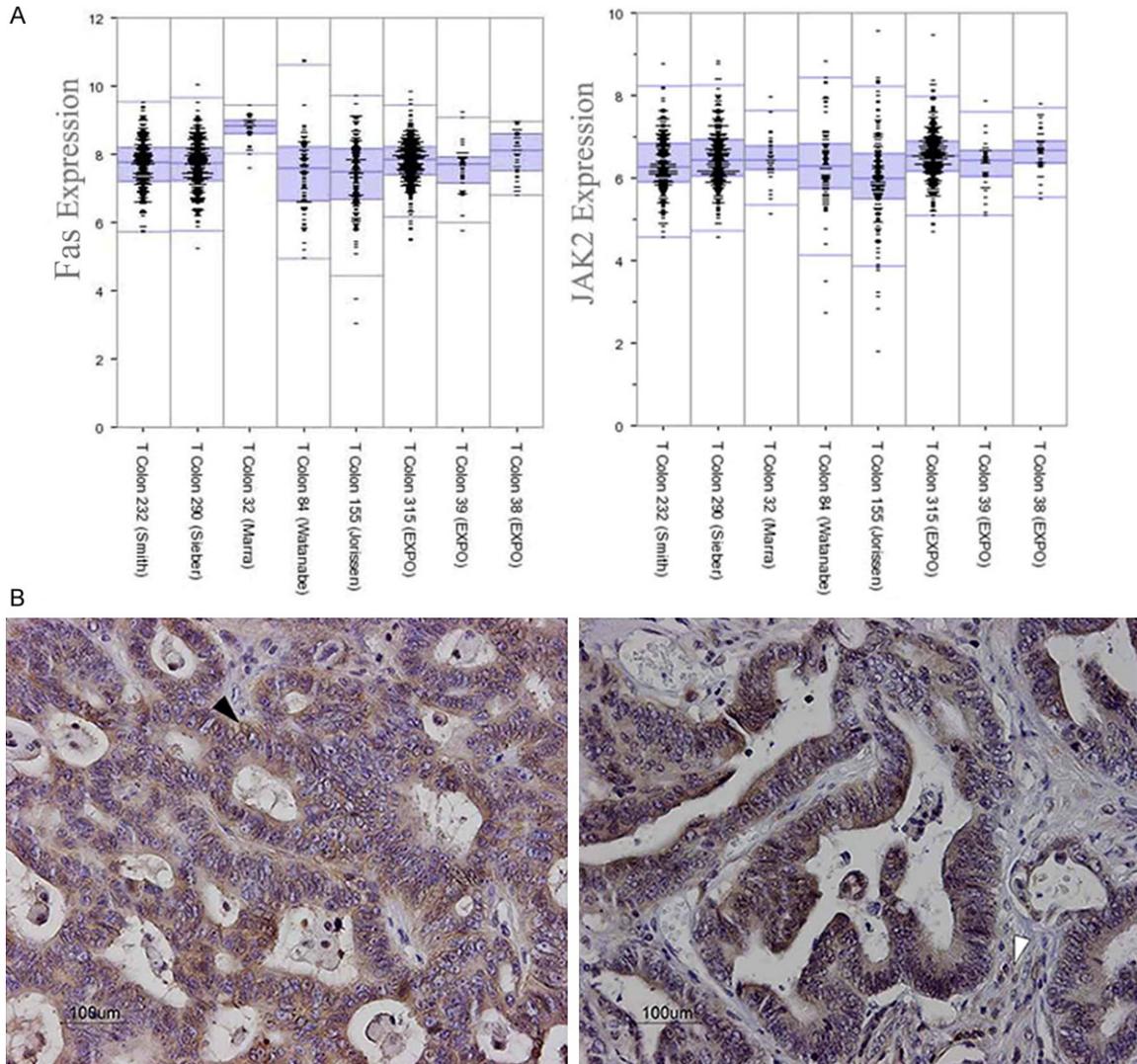
### *Clone formation assay*

Cell cultures medium was refreshed one day before clone formation assay. Immediately before clone formation assay, cell cultures were collected in 50 ml sterilized tubes (BD Falcon). After spheroids sink down, supernatant was aspirated and then 1-2 ml Cell Aggregate Dissociation Medium (eBioscience) added. Spheroids were resuspended in dissociation medium and the tube was incubated in 37°C water bath. Resuspend with a sterilized pipette every five minutes until spheroids were dissociated completely. Let the dissociated suspension go through 40 µm sterilized cell strainer (Becton Dickinson) and collect single cells from below the strainer. Spinned down and re-suspended single-cell suspensions to make a density of 20 cells per µl in culture medium. Gently pipetted a mixture of 50 µl single cell suspension and 50 µl thawed-on-ice liquid Matrigel and seeded onto a well of 6-well or 12-well plates (Costar). Incubate the 6-well or 12-well plates in 37°C for 10 min to make Matrigel hard. The appropriate volume of culture medium was applied on Matrigel. Cultures were fed every 3 days and tumor sphere formation was monitored weekly. Under 10× or 40× magnification of inverted phase-contrast microscope (EVOS, AMG, USA), images were acquired to visualize the morphology of sphere, and all spheres were counted, plotted and presented as histogram. Each experiment was performed in triplicate. At least two independent experiments were repeated.

### *Western blot*

CRC cells were lysed to get total proteins for each cell line using phosphatase and protease inhibitor diluted in RAS lysis buffer/glycerin phosphate. Protein concentration was analyzed using Bio-Rad protein assay based on Bradford

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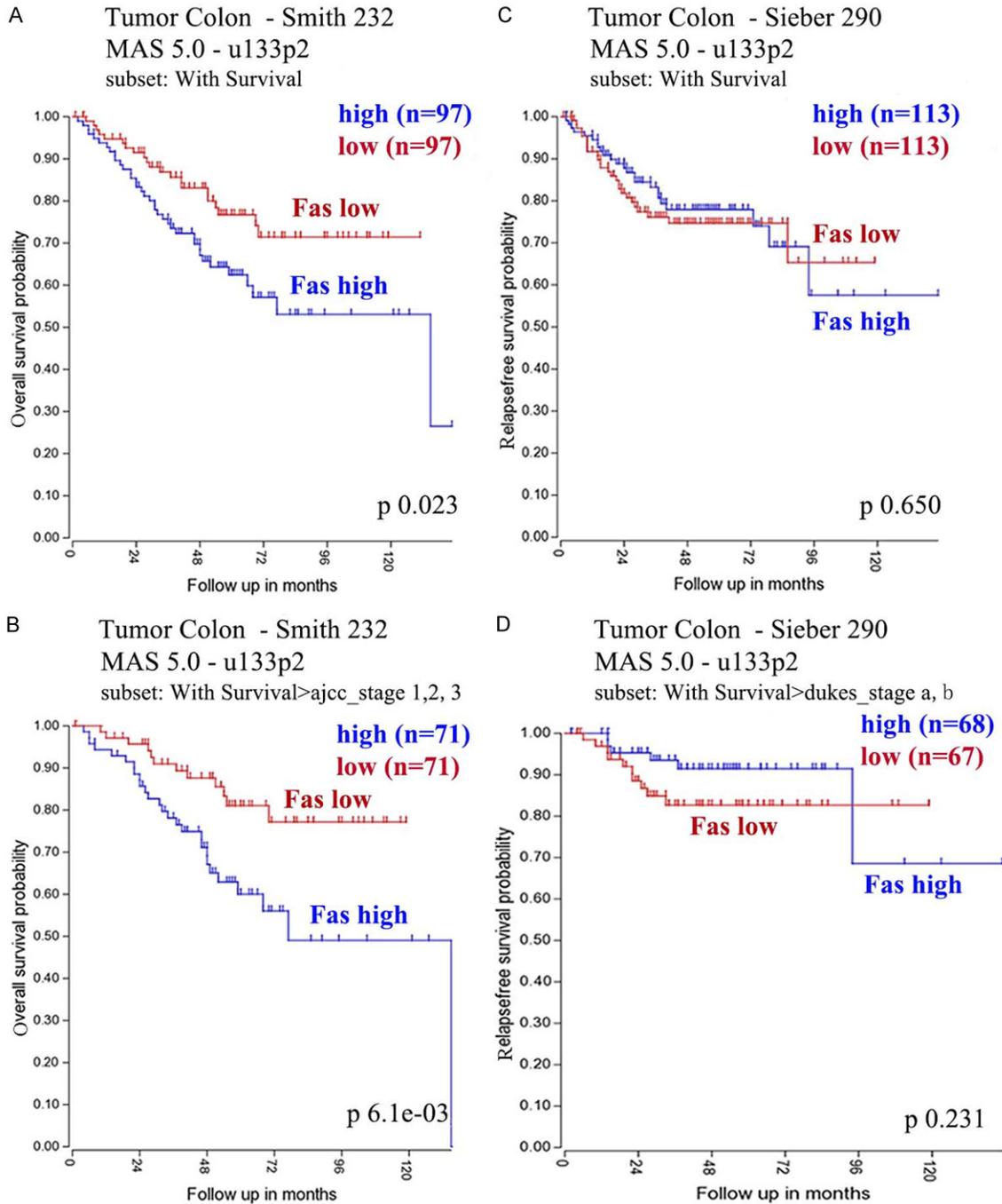


**Figure 1.** mRNA levels of FAS/FAS and JAK2 in all tumors from 8 different cohorts. A. For each cohort, total RNA was extracted from retrieved fresh-frozen colorectal cancer specimens, then labeled and hybridized to HG-U133Plus2.0 GeneChip arrays. Signal intensities on the chip were normalized using the quantile normalization procedure implemented in robust multi-array analysis (RMA) [20] and the normalized data were transformed to log form (Y axis), which reflects RNA level. Here, mRNA levels of FAS and JAK2 in colorectal cancer specimens from different cohorts were quantified and plotted. FAS and JAK2 were expressed at considerable levels in these cohorts. The average levels of JAK2 among these cohorts were consistent. B. Immunohistochemistry analysis of FAS expression in colorectal cancer tissues. FAS is widely expressed in both cell membrane and cytoplasm in colorectal tumor cell, and also in a few stromal tissue. Black arrowhead shows the examples of expression in tumor cell. White arrowhead shows expression in stromal tissue.

method. The protein measurement was done by a microplate reader at 595 nm wavelength. Comparison to a standard curve provides a relative protein concentration. Load 40 µg of total protein for SDS-PAGE electrophoresis, and then transfer to PVDF membrane. Protein bands can be visualized by ponceau S staining. For FAS and actin, block with TBS-0.1% Tween-5% non-fat milk; for JAK2, block with TBS-0.1% Tween-

5% BSA. Blocking procedure requires rotating at room temperature 2 hours. Wash with TBS-0.1% Tween, and then add rabbit anti-human-FAS antibody (1:500), rabbit anti-human-JAK2 antibody (1:1000) and actin on separate transferred PVDF membranes. Incubate at 4°C overnight and wash with TBS-0.1% Tween 3 times, 10 min for each. The PVDF membranes were probed with goat anti-rabbit HRP-labeled sec-

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**Figure 2.** FAS expression in primary CRC is associated with shorter overall survival (OS) in non-metastatic colorectal cancer. Expression level of FAS in a cohort of 232 primary CRCs [16] (left upper and lower panel) and a cohort of 290 primary CRCs [17] (right upper and lower panel). Median expression was used as a cutoff. A. Kaplan-Meier curves show that expression of FAS is associated with a significantly shorter OS. B. Kaplan-Meier curves indicate that, except for stage 4 colorectal cancer, high FAS significantly correlates with shorter OS. C and D. Kaplan-Meier curves show that high FAS correlates with shorter survival in survival cases, however, the correlation in cohort of 290 primary CRCs is not significant.

ondary antibody. Detect with ECL detection agent and expose the film. Scan the images and

quantify the intensity using Bio-Rad Quantity One analyzing software.

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**Table 1.** FAS associated pathways by KEGG pathway finder

KEGG pathway	P value (Smith et al)	P value (Sieber et al)	P value (Combined)
Chemokine signaling pathway	1.0e-05	3.1e-05	3.5e-09
Antigen processing and presentation	6.9e-05	9.5e-06	5.3e-09
Apoptosis	4.5e-03	0.04	1.4e-03
ECM receptor interaction	9.2e-04	4.0e-04	1.3e-06
T cell receptor signaling pathway	2.1e-04	9.7e-03	1.5e-05
Cytokine cytokine receptor interaction	1.1e-03	9.7e-03	5.0e-05

FAS-associated pathways in each separate tumor cohort were analyzed using KEGG pathways, which shows pathways significantly associated with FAS expression in two data sets. Combined P values were calculated using the web tool Meta P software.

**Table 2.** FAS associated pathways by Gene Ontology (GO)

GO	P value (Smith et al)	P value (Sieber et al)	P value (Combined)
Immune Response	4.4e-69	5.1e-70	1.3e-135
Antigen processing and presentation	7.6e-259	1.4e-272	2.8 e-509
Inflammatory response	1.6e-134	1.0e-140	1.8 e-270

FAS-associated pathways in each separate tumor cohort were analyzed using GO terms function in R2. Combined P values were calculated using the web tool Meta P software.

showed both tumor membrane and cytoplasm staining with moderate to strong staining in 8 of 10 CRC patients (**Figure 1B**). Then, survival curve was done to assess whether FAS expression would associate with clinical outcome. Using R2 database, FAS associated survival curves were done on several CRC cohorts which demonstrated high FAS expression in primary CRC correlated with poor prognosis (**Figure 2**).

*JAK2 is in association with FAS expression and correlates with poor survival*

### Statistical analysis

Correlation analysis between FAS and JAK2 expression was performed by choosing 'linear analysis' function in GraphPad Prime 5.0 software. Differences between groups were analyzed using 'unpaired two-sided T-test' function in GraphPad Prime 5.0 software. Data are expressed as means  $\pm$  SD. P-value < 0.05 was considered as statistically significant.

### Results

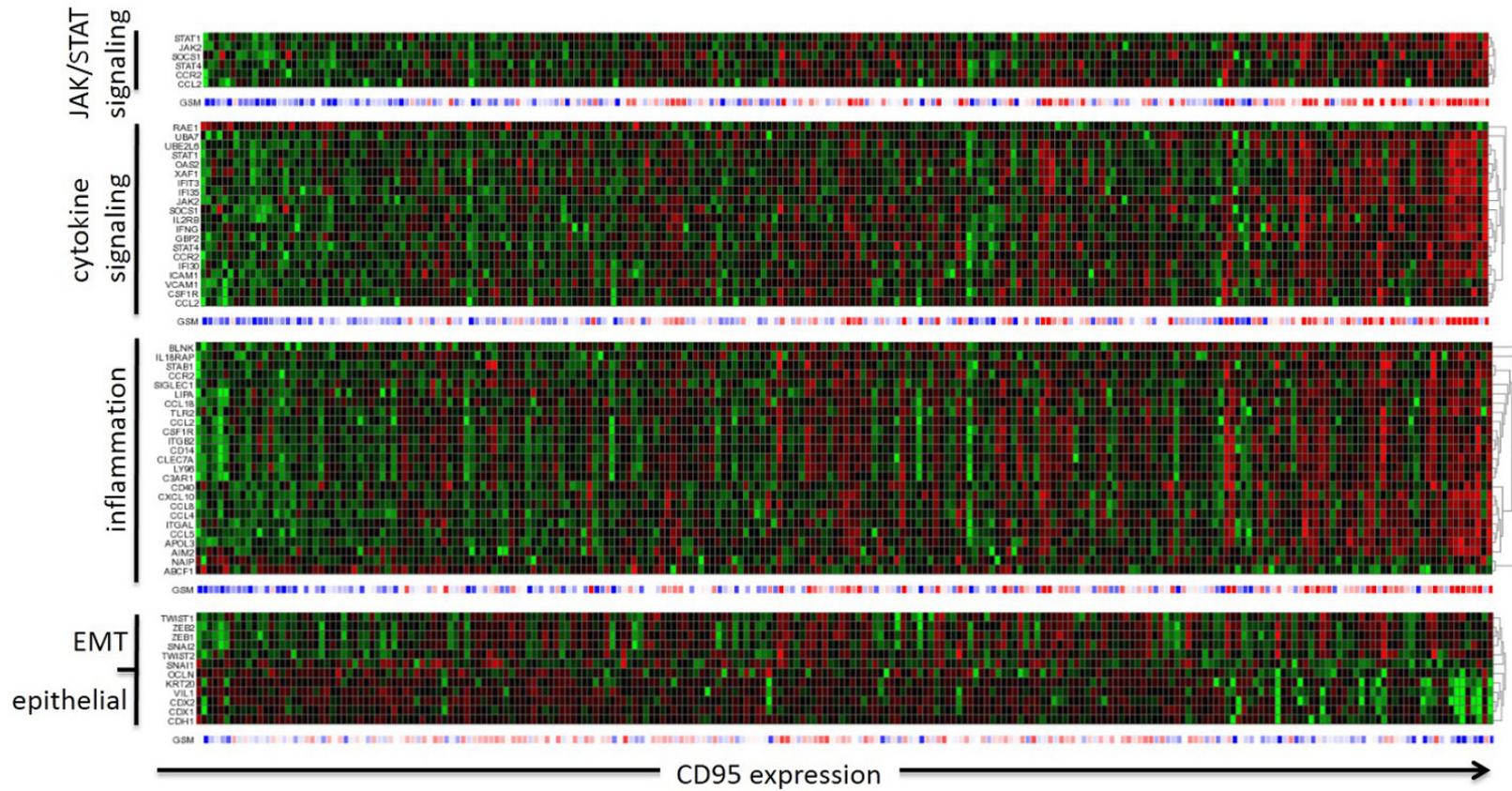
*High FAS expression is associated with shorter survival in non-metastatic colorectal cancers (CRC)*

Our previous results showed that the death receptor FAS stimulates tumor cell invasion *in vitro* [19]. However, little is known about clinical outcome of high FAS-expressing colorectal cancers. Thus, we first analyzed FAS mRNA levels in eight separate CRC patient cohorts in order to investigate if FAS expression would correlate with CRCs prognosis. Although direct comparison of expression levels between FAS and JAK2 is not available based on mRNA microarray data, it is shown that both FAS and JAK2 are expressed at relatively high level in most colorectal tumors (**Figure 1A**). Immunohistochemical staining for FAS in CRC sections

FAS-centered bioinformatics study was performed first by "KEGG Pathway Finder" function in R2 database. We searched for FAS-associated cellular processes, pathways, associated genes in two data sets from two separate CRC cohorts. The KEGG pathway analysis reveals several pathways that were significantly associated with FAS expression level in the two data sets (**Table 1**). Gene Ontology analysis also reveals that the pathways in association with FAS are functionally involved in immune response, antigen processing and presentation, inflammatory response (**Table 2**).

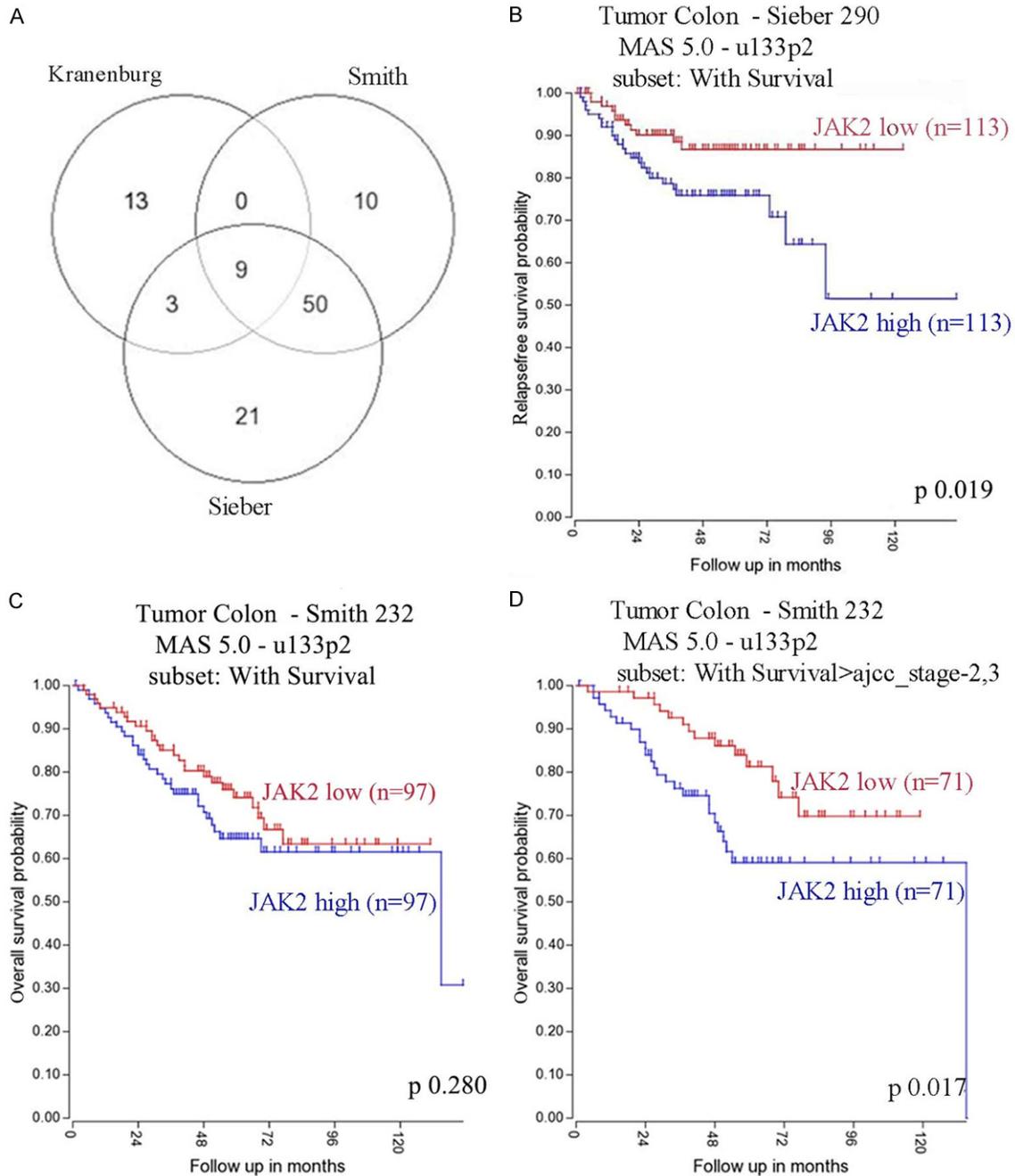
The lists of genes involved in the above processes were generated by clicking each pathway in R2. Then string analysis was performed by importing each list of genes into string web application tool to get visualized interconnections for proteins. Gene network of the immune response pathway is functionally characterized by INF- $\gamma$  and chemokine signaling (data not shown), which to a large extent depends on JAK2-mediated (data not shown). The proteins in the chemokine signaling network are also interconnected in inflammatory response (data not shown). All of the genes that positively correlated with FAS were shown in heat maps where the tumors were ordered according to FAS expression levels from low to high (**Figure 3**). They governed chemokine signaling, JAK/

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**Figure 3.** Heatmap analysis for FAS-associated genes and kinases. Heat map lists FAS associated genes in three colorectal cancer cohorts (Smith et al [16], Sieber et al [17], Kranenburg et al [24]) according to FAS expression in the categories including JAK/STAT signaling, cytokine signaling, inflammation, and EMT.

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**Figure 4.** FAS-associated genes overlap and JAK2 related Kaplan-Meier curve in different cohorts. A. Overlap between FAS-associated kinases from the three cohorts was identified by GeneVenn [25]. B-D. JAK2 expression in primary CRC is associated with shorter overall survival (OS) in non-metastatic colorectal cancer. Expression level of JAK2 in a cohort of 290 primary CRCs (right upper panel) and a cohort of 232 primary CRCs (lower panels). Median expression was used as a cutoff. C. Kaplan-Meier curve shows that expression of JAK2 is associated with a significantly shorter OS. D. Kaplan-Meier curve suggests that in stage 2 and stage 3 CRCs, high JAK2 significantly correlates with shorter OS.

STAT signaling, inflammation and epithelial-mesenchymal transition (EMT). Besides, GeneVenn was performed to three separate CRC cohorts and found 9 genes encoding kinases that were significantly associated with FAS ( $P <$

0.01), among which JAK2 was on top with combined  $p$  value  $2.8e-34$  (Figure 4A; Table 3).

As a member of JAKs, JAK2/STAT3 seems to be an important mediator of chemokine (such as

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**Table 3.** Kinases positively associated with FAS

Kinase	Sieber		Smith		Kranenburg		Combined	
	R value	P value	R value	P value	R value	P value	R value	P value
JAK2	0.458	2.07e-14	0.535	4.18e-16	0.557	3.10e-08	0.517	2.80e-34
SGK1	0.363	7.64e-09	0.394	2.23e-08	0.312	9.52e-03	0.356	4.00e-17
ITK	0.328	2.55e-07	0.381	6.72e-08	0.485	6.90e-06	0.398	4.10e-17
DAPK1	0.307	1.72e-06	0.323	7.10e-06	0.380	7.95e-04	0.337	4.20e-13
NAGK	0.281	1.26e-05	0.252	6.37e-04	0.453	3.63e-05	0.329	6.40e-11
CSF1R	0.219	8.54e-04	0.307	2.05e-05	0.467	1.80e-05	0.331	2.70e-10
DGKA	0.308	1.65e-06	0.236	0.001353	0.331	0.005545	0.292	2.70e-10
CMPK2	0.252	0.00011	0.3	3.24e-05	0.341	0.003741	0.298	4.70e-10
MAP3K6	0.199	0.002656	0.224	0.002408	0.354	0.002294	0.259	7.60e-07

FAS-associated kinases are shown with R and P value in each data sets. Combined R and P values were calculated with the Web-based Meta P software.

IL-6) signal in promoting tumor genesis and metastatic progression [22]. Persistent activation of JAK/STAT signaling is implicated in not only tumor genesis but also tumor survival and metastasis [23]. In order to see whether JAK2 expression would correlate with poor survival, we analyzed JAK2 expression in two separate CRC cohorts in a Kaplan Meier curve, and found that high JAK2 expression associate with poor disease free survival (DFS) and overall survival (OS) (**Figure 4B-D**).

*JAK2 has good correlation with FAS both in patients' slides and cell lines*

The above gene microarray analysis and bioinformatics study link JAK2 expression to D95 expression in tumor local immune response, chemokine signaling and CRC progression or metastasis. To test if there was confident correlation between JAK2 and FAS, we performed IHC on CRC paraffin-embedded sections and western blots using patient originated cell lines CRC 29, CRC47, CR9, CR16, CRC48, CRC26, and liver metastatic cell lines L169, L167, L145. IHC images suggest that CRC tumor tissue with high FAS expression also express high level of JAK2, and vice versa (**Figure 5A**). Linear analysis for JAK2 and FAS expression reveals JAK2 and FAS expression have good correlation (**Figure 5B**). Next, we lysed CRC cells and performed western blot. We probed western blots (WB) with JAK2 and FAS antibody followed by secondary HRP-conjugated antibody. WB show that both proteins express in majority CRC cell lines at various levels. Using quantity one (Bio rad) software, the protein expression levels on WB were quantified in intensity form

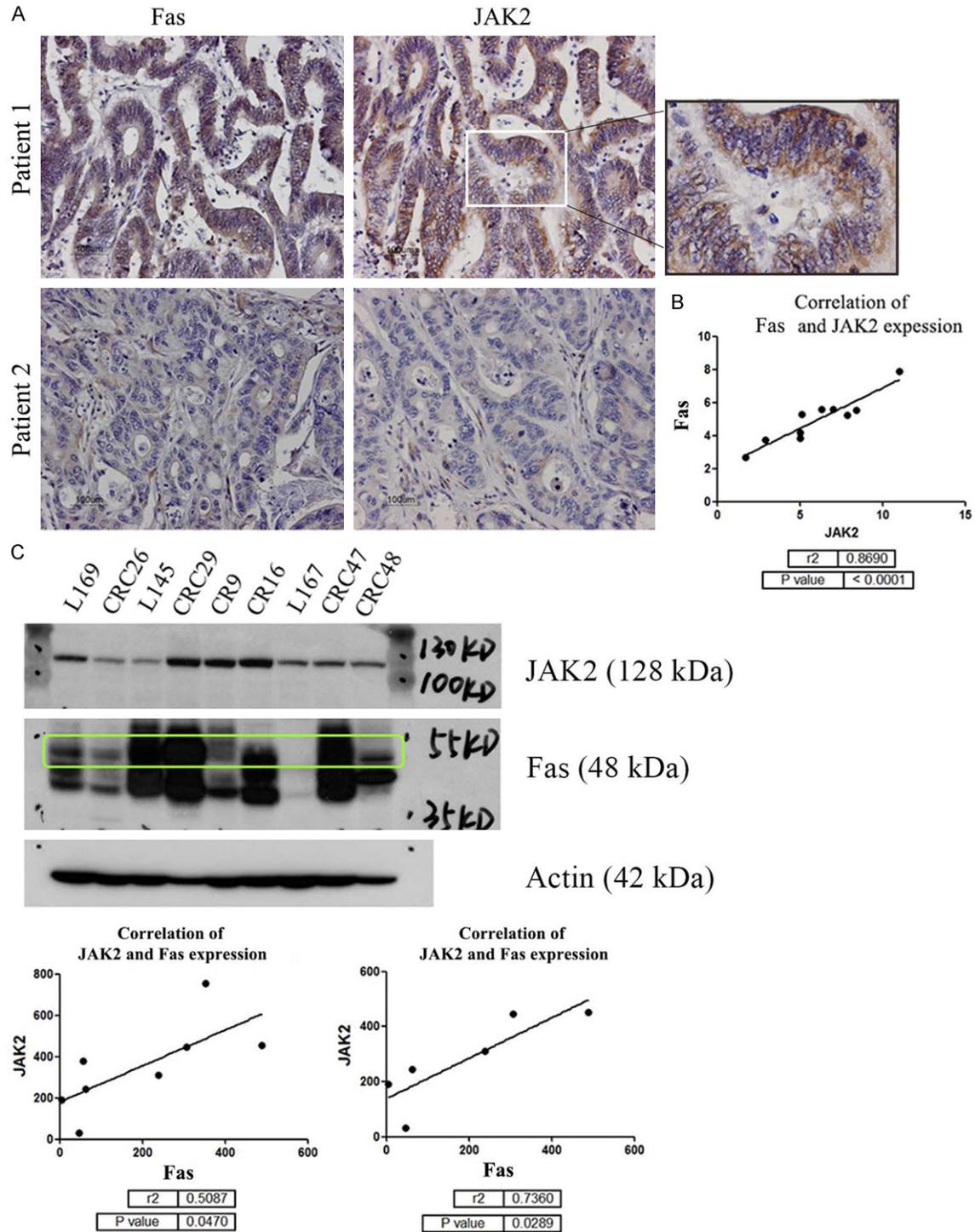
int × mm, and correlation analysis links JAK2 to FAS (P-value < 0.05, r=0.86) (**Figure 5C**).

*JAK2 inhibition suppresses CRC cells clone formation capacity in vitro*

JAK2 was first found as a constitutively activated tyrosine kinase in many myeloproliferative neoplasms (MPNs) [26, 27]. Since high JAK2 expression correlates shorter survival in CRC, we presumed that JAK2 might contribute to CRC cell proliferation. In order to evaluate the role of JAK2 in CRC tumor genesis, single cells were seeded in Matrigel supplemented with culture medium, with or without CEP33779 (JAK2 specific inhibitor) treatment. Here, each condition was done in triplicates. Each experiment was performed three times separately. A significant reduction in number of tumor clones were detected in CEP33779 treated group in dose dependent manner, compared with DMSO treated group (**Figure 6**). Besides, tumor clones in CEP33779 environment was much smaller than those in DMSO environment. These suggest that JAK2 inhibition significantly suppress CRC cells clone formation capacity.

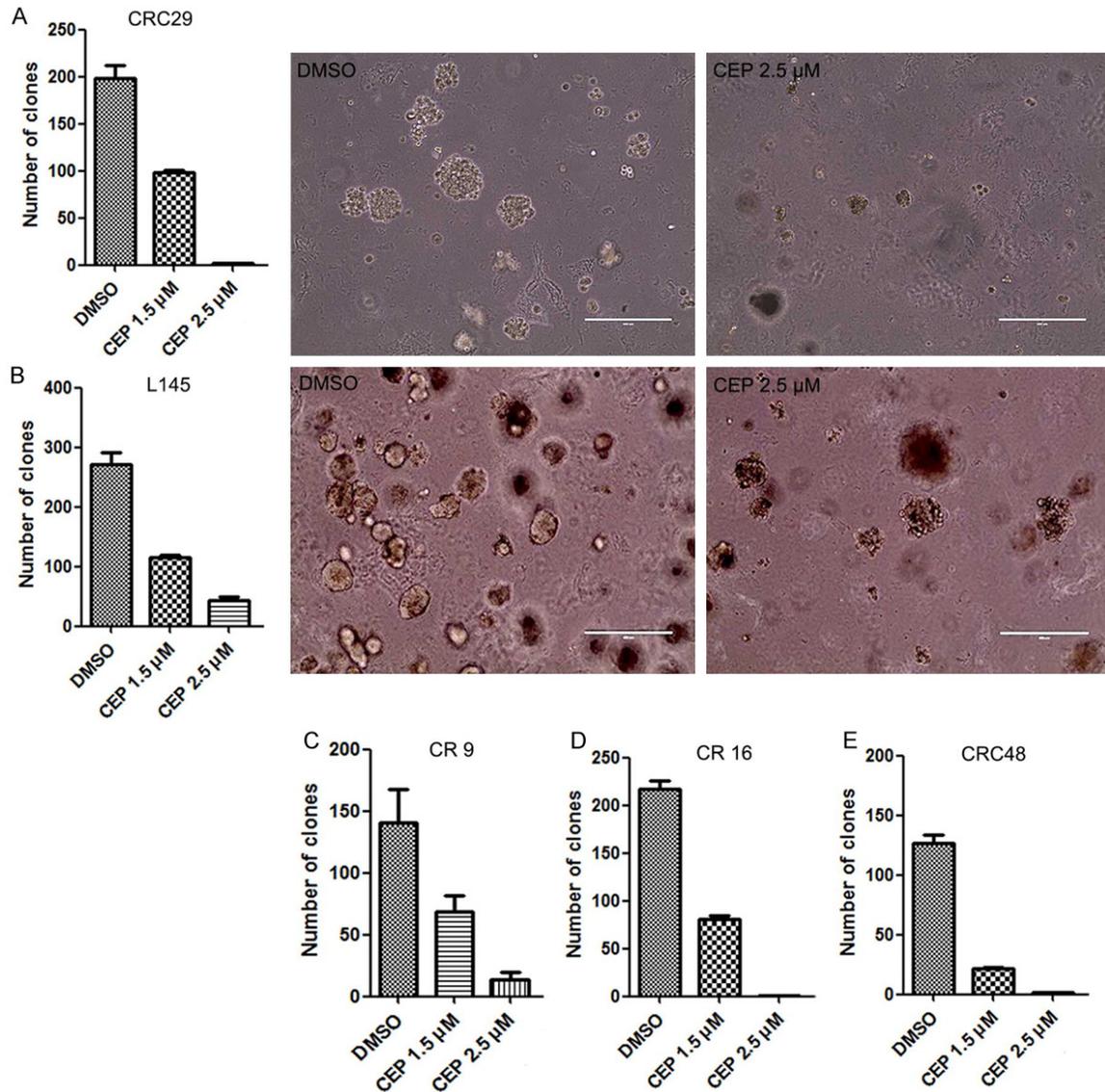
### Discussion

Finding potential markers for predicting outcomes of colorectal cancer patients would make it of great value for the clinic to seek out other possible target treatment. In this article, we present evidence that colorectal cancer (CRC) tissue express JAK2 and FAS which significantly associate with disease free survival (DFS) and overall survival (OS) in non-metastasis CRC patients.



**Figure 5.** Correlation of JAK2 and FAS in CRC primary tumors and CRC cell lines. A. Examples of immunohistochemistry pictures show FAS (left two panels) and JAK2 (right two panels) expression in two patients. It is obvious that patient 1 expresses low FAS (left upper panel) and JAK2 (right upper panel), and vice versa in patient 2 (left and right lower panels). JAK2 is mainly expressed in cytoplasm in colorectal tumor cells. B. Correlation analysis of JAK2 and FAS expression scores. Immunohistochemistry for JAK2 and FAS of 10 CRC patients was done followed by normalized scoring of each slide, intensity and density. C. Expression of JAK2 and FAS in the above cell lines. The bands on western blot were quantified using quantity one analyzing tool and were then input in Graphpad Prime 5.0 statistic software to plot their correlation. Correlation of JAK2 and FAS in all cell lines was plotted in left lower panel. Correlation of JAK2 and FAS in primary tumors (metastasis excluded) was plotted in right lower panel.

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**Figure 6.** Clone formation capacity was assayed in different cell lines CRC29, L145, CR9, CR16, and CRC48. A-E. For each cell line, single cell was seeded in 12-well plate in a density of 1000 cells/100  $\mu$ l Matrigel & culture medium, cultured in medium with 2.5  $\mu$ M DMSO (DMSO), 1.5  $\mu$ M CEP33779 (CEP 1.5  $\mu$ M), 2.5  $\mu$ M CEP33779 (CEP 2.5  $\mu$ M). The DMSO group is considered as control. Each experiment was done in triplicates. Graphs show the average number of clones per 1000 single cells in each condition. CEP33779 in a concentration of 1.5  $\mu$ M can suppresses clone formation capacity, and the suppression effect is even stronger at higher concentration (CEP 2.5  $\mu$ M) ( $P$ -value < 0.05). A, B. Pictures are the examples of clones in DMSO and CEP 2.5  $\mu$ M group taken under inverted microscope in 10X magnification.

JAK2 is a member of the Janus kinase (JAK) family, which also includes JAK1, JAK3 and TYK2. JAK2 is a non-receptor tyrosine kinase which is widely expressed and residue mostly in cytoplasm, functionally mediating cytokine induced inflammation. Aberrant JAK2 signaling was found in myeloproliferative neoplasms (MPNs). Given that some colorectal tumors are associated with inflammation, researchers

found that JAK2 may to some degree contribute to colitis-induced colorectal cancer in a mouse model [28]. In the present study, we found that JAK2 expression level is moderate to high in separate cohorts and high JAK2 expression correlates with shorter DFS and OS, suggesting that JAK2 may play a positive role in tumor progression and invasion. By using 'KEGG pathway finder' software, we analyzed

the correlation between JAK2 and FAS which provide evidence of the correlation. This is consistent with CRC cell cultures *in vitro*. Thus, we assume that JAK2 may help tumor growth in FAS expressing CRCs.

Tumor progression and metastasis are two main cause of poor prognosis. In previous study, we found that FAS contributed to CRC cells invasive behavior via cofilin pathway [29]. In some mouse models of CRC liver metastasis, loss of FAS reduces liver metastasis [30, 31]. And except for inducing apoptosis, FAS can also promote tumorigenesis [32], possibly by activating non-apoptotic signaling pathways that stimulate tumor cell proliferation, invasion and metastasis [33, 34]. Interestingly, at least in part, this process depends on FLIP, possibly switching FAS signaling from apoptotic to non-apoptotic JNK or ERK/NFκB signaling [35] instead of FADD caspases activation. Furthermore, JAK2 inhibition impairs clone forming capacity of CRC cells, suggesting that JAK2 inhibition blocks surviving pathways including FAS pathway.

In conclusion, this study suggests that JAK2 and FAS are two of the most important molecules in CRC tumor progression, and aberrant expression of both proteins are significantly associated with DFS and OS in non-metastatic CRCs. These data contribute to future improvement in prognosis for patients with colorectal cancer and provide potential target for antitumor therapy. Up to day, there are only limited reports with respect to using JAK2 and FAS as prognostic factor in predicting outcomes for CRC patients after surgery. Therefore, large population-based research is required and more *in vitro* and *in vivo* experiments are needed to support our findings.

### Translational relevance

Despite the application of commonly used biomarkers in predicting early stage colorectal cancers (CRCs), a growing amount of CRCs are detected and a large population of advanced CRC patients (stage II/III) are in high risk of relapse and metastasis. Therefore, novel biomarkers which are significantly associated to tumor progression are needed to predict survival. Thus, the patients in poor prognosis can take proper treatment in time. In this study, we show that JAK2 expression in human colorectal

cancers is strongly correlated with FAS expression, patients' survival, especially in non-metastatic colorectal cancers. We also found that JAK2 plays an important role in FAS associated gene networks. We further show JAK2 inhibition can suppress tumor colony formation in cell cultures by using JAK2 specific inhibitors working in medium/Matrigel culturing system. Importantly, high JAK2 expression levels were found to be correlated with poor overall survival in advanced CRC patients. Thus, these results provide preliminary value to support the further evaluation of JAK2 and FAS as a prognostic biomarker and novel therapeutic targets in CRCs.

### Disclosure of conflict of interest

None.

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