Published in final edited form as:

Clin Colorectal Cancer. 2018 December ; 17(4): e741–e749. doi:10.1016/j.clcc.2018.08.002.

Immune-related genes to dominate neutrophil-lymphocyte ratio (NLR) associated with survival of cetuximab treatment in metastatic colorectal cancer

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Conflict of Interest

Author Y.S. has received honoraria from Taiho Pharmaceutical, Chugai Pharma, Yakult Honsha, Takeda, Merck Serono, Bayer Yakuhin, Eli Lilly Japan, and Sanofi. A.T. has received honoraria from Daiichi Sankyo, Taiho Pharmaceutical, Chugai Pharma, Merck Serono, Takeda Pharmaceutical, and Bristol-Myers Squibb Japan; Speakers' Bureau from Chugai Pharma, Taiho Pharmaceutical, Takeda, and Merck Serono. W.I. has received honoraria from Chugai Pharma, Merck Serono, Takeda Pharmaceutical, and Taiho Pharmaceutical; research funding from Chugai Pharma, Takeda Pharmaceutical, and Taiho Pharmaceutical. H-J.L. discloses consulting or advisory role for Merck Serono, Roche, Bayer, and Pfizer, and travel expenses for Merck Serono, Bayer, and Roche, and honoraria for Merck Serono, Roche, Bayer, and Boehringer Ingelheim. The other authors have declared no conflicts of interest.

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Abstract

Purpose: Few clinical studies have investigated the association between neutrophil-lymphocyte ratio (NLR) and treatment with cetuximab-based chemotherapy in metastatic colorectal cancer (mCRC). The NLR may reflect immune cells modulating specific cytokine signals in the tumor microenvironment; however, which immune-related genes affect the NLR remain unclear.

Patients and Methods: In 77 patients with *KRAS* exon2 wild-type mCRC from prospective trials of 1st-line chemotherapy with cetuximab, expression levels of 354 immune-related genes were measured in tissue samples obtained from all patients by the *HTG EdgeSeq Oncology Biomarker Panel*. The association between the NLR and clinical outcomes was evaluated using Spearman's rank correlation coefficient. In addition, two-sample t-tests were performed to investigate which genes among the top 100 genes associated with survival had significantly different expression levels between the NLR-low and NLR-high groups among all measured genes.

Results: NLR data were available for 71 patients. The NLR was associated with progression-free survival (PFS) and overall survival (OS) ($r=-0.24$; $p=0.040$ and $r=-0.29$; $p=0.010$, respectively). When stratified by the median value of the NLR, the Kaplan-Meier curve of NLR-low vs. NLR-high differed significantly for both PFS (median 11.8 vs. 9.1 m, $p=0.036$) and OS (median 42.8 vs. 26.7 m, $p=0.029$). The two-sample t-test revealed that the expression levels of the *LYZ*, *TYMP*, and *CD68* genes differed significantly between the NLR-low and NLR-high groups (t-test p -value <0.005 , FDR p -value <0.15).

Conclusion: NLR is significantly associated with survival in mCRC patients treated with 1st-line chemotherapy with cetuximab. Genes encoding for activities on macrophages may affect the NLR.

Micro-Abstract

Our study using data of prospective trials demonstrated that the neutrophil-lymphocyte ratio (NLR) was associated with survival time in *KRAS* wild-type metastatic colorectal cancer patients treated with 1st-line chemotherapy with cetuximab. The expression levels of the *LYZ*, *TYMP*, and *CD68* genes differed significantly between the NLR-low and NLR-high groups. Genes encoding for activities on macrophages may affect the NLR.

Keywords

colorectal cancer; neutrophil-lymphocyte ratio; cetuximab; prognostic marker; macrophages

Introduction

The neutrophil-to-lymphocyte ratio (NLR) is a marker of inflammation, and an elevated NLR reflects enhanced systemic inflammation, and is associated with reduced tumor-specific immunity such as decreased tumor-infiltrating lymphocytes (TILs) in the tumor.¹ Neutrophils, monocytes, and platelets have been reported to promote tumor development via different mechanisms, whereas lymphocytes are essential for the elimination of cancer cells.² Distinct tumor suppressive-mechanisms are mainly controlled by CD4 and CD8 T lymphocytes, but regulatory T cell contributes to tumor-induced immunotolerance through suppression of the CD4 T lymphocyte. Various cytokines mediate the attraction of immune cells in the tumor microenvironment. A high NLR has been shown to correlate with a distinct cytokine profile including pro-inflammatory cytokines and angiogenic cytokines which are related to key biological processes involved in carcinogenesis.³ This may partly explain why an elevated NLR is associated with poor outcomes in cancer.

A systematic review and meta-analysis of 100 studies including 40,559 patients with various solid tumors indicated that NLR was associated with poor survival in all disease subgroups, sites, and stages. Meta-analyses and systemic reviews based on relevant studies showed that NLR has prognostic value in patients with colorectal cancer (CRC). An elevated pretreatment NLR predicted poorer survival in CRC.^{4–6} The role of pre-treatment inflammatory indices in predicting the outcomes of patients with CRC has been clearly evidenced in patients with radically resected tumors⁷ and was more recently suggested in patients with metastatic disease.^{8–11} The NLR was a better independent predictor of outcomes than was the lymphocyte-to-monocyte ratio, platelet-to-lymphocyte ratio, and prognostic nutritional index in patients with CRC.¹²

A significant association of the NLR with clinical outcomes has been reported in several retrospective studies of patients with mCRC treated with chemotherapy plus bevacizumab.^{13,14} A retrospective study of a large prospective trial, TRIBE, validated the prognostic value of the NLR in patients with mCRC who received intensive chemotherapy with bevacizumab.¹⁴ However, few clinical studies have investigated the association between the NLR and treatment with cetuximab-based chemotherapy. The NLR may reflect immune cells modulating specific cytokine signals in the tumor microenvironment; however, which immune-related molecular signals affect the NLR remain unclear. We therefore performed a biomarker study to evaluate association between the NLR and the clinical outcomes of cetuximab treatment for mCRC and to investigate which immune-related genes significantly affect the NLR.

Patients and Methods

Study design and patient population

We conducted a retrospective study including patients with *KRAS* exon2 wild-type mCRC whose tissue samples were available for measurement of gene expression levels, from 2 prospective clinical trials evaluating the combination of cetuximab and oxaliplatin-based chemotherapy as 1st-line treatment, modified-FOLFOX6 (JACCRO CC-05 trial: N=28/57, UMIN000004197), and SOX (JACCRO CC-06 trial: N=49/67, UMIN000007022). The identical eligibility criteria of the 2 trials were as follows: adenocarcinoma of the colon or rectum with immunohistological expression of EGFR; *KRAS* exon 2 wild-type tumor with unresectable metastases; at least one measurable lesion of 10mm or a residual nonmeasurable lesion according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1; adequate bone marrow function, hepatic function, and renal function; an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 or 1; and an age of 20 to 79 years. Patients with uncontrolled infection, massive ascites or pleural effusion, symptomatic brain metastases, or other malignancies within 5 years before enrollment (with the exception of early carcinoma that had been treated with curative intent), a history of systemic chemotherapy for mCRC, or who had previously received oxaliplatin or cetuximab were excluded. This biomarker study was conducted in accordance with the Declaration of Helsinki and was approved by the ethical committee of each participating institution. Written informed consent was obtained from all patients before enrollment.

Chemotherapy

In the JACCRO CC-05 trial, patients received modified-FOLFOX6 [oxaliplatin in a dose of 85mg/m² of body surface area (BSA); leucovorin (LV) in a dose of 200 mg/m² of BSA; an intravenous bolus of fluorouracil (FU) in a dose of 400 mg/m² of BSA; a continuous 46-h infusion of FU in a dose of 2400 mg/m² of BSA] on day 1 of each 14-day treatment cycle plus cetuximab (a loading dose of 400 mg/m² of BSA, followed by 250 mg/m² of BSA given weekly thereafter). In the JACCRO CC-06 trial, patients received SOX (a 120-min infusion of oxaliplatin in a dose of 130 mg/m² of BSA on day 1 plus oral S-1 in a dose of 80 mg/m² of BSA on days 1–15) on day 1 of each 21-day treatment cycle plus cetuximab. In both trials, treatment was continued until disease progression, unacceptable toxic effects developed, a complete response was achieved, surgical resection became possible, or the patient requested or the physician decided that therapy should be withdrawn.

Assessment of efficacy

The primary endpoint of the 2 phase II trials was the objective response rate (ORR) (complete or partial response). Secondary endpoints included progression-free survival (PFS), based on disease progression as detected by external review or death from any cause, overall survival (OS), secondary resection of metastases with curative intent, and safety. Responses were evaluated according to the Response Evaluation Criteria in Solid Tumors, version 1.1 by the investigators and were then validated by an external review board.

RNA isolation and gene expression analysis

Formalin-fixed, paraffin-embedded (FFPE) tumor specimens were cut into sections with a thickness of 3 or 10 μm . In a preparation for macrodissection, one 3- μm slide was stained with hematoxylin and eosin and was then evaluated for tumor content and marked for areas with dominant tumor foci by a pathologist. Macrodissection was performed for 10- μm slides by scrapping the marked areas with a blade to ensure that as many tumor cells as possible were dissected. Total RNA was extracted from FFPE tissue of the tumor samples on 10- μm slides, using an miRNeasy FFPE Kit (QIAGEN KK, Tokyo, Japan) according to the manufacturer's protocol.

Immune-related gene expression levels were measured with the use of an *HTG EdgeSeq Oncology Biomarker Panel*, which is comprised of probes targeting 354 genes implicated in the host immune response to tumors. Next generation sequencing was used to quantitatively analyze targeted genes (<https://www.htgmolecular.com/assays/io>).

DNA isolation and BRAF mutation analysis

Genomic DNA was extracted from the FFPE tissue derived from the tumor samples with the use of a QIAamp DNA FFPE Tissue Kit (QIAGEN KK) according to the manufacturer's protocol. *BRAF*V600E mutations were detected by dye terminator sequencing. Exon 15 of the *BRAF* gene was amplified by polymerase chain reaction (PCR), and the PCR products were then visualized using agarose gel electrophoresis with ethidium bromide staining. The products were directly sequenced with the use of an ABI 3130xl Genetic Analyzer (Thermo Fisher Scientific K.K., Yokohama, Japan) according to the manufacturer's instructions.

This study was conducted in accordance with the REporting recommendations for tumor MARKer prognostic studies (REMARK).¹⁵ Tissue analyses were performed at HTG Molecular, Inc. (Tucson, AZ, United States) after obtaining approval from the Institutional Review Board of each institution that participated in the JACCRO CC-05/06AR trials (UMIN000010635).

Statistical evaluation

Univariate Cox regression analysis was conducted in cases that passed the internal Quality Control metrics of the *HTG EdgeSeq Oncology Biomarker Panel*. The association between NLR and PFS and OS in the entire cohort was evaluated using the Spearman's rank correlation coefficient. The median of NLR expression was used as the cutoff to stratify patients to NLR-low and NLR-high groups. The association between NLR-low vs high groups and clinical outcomes was evaluated using the Fisher's exact test for ORR, and the log-rank test for PFS and OS. The multivariable Cox proportional-hazards regression model was fitted to reevaluate the association between NLR groups with PFS and OS adjusting for ECOG PS, the number of organs involved, and tumor location. Tumors located from the cecum to the splenic flexure were classified as right-sided, while tumors that involved the splenic flexure, descending colon, sigmoid colon, and rectum were classified as left-sided. The two-sample t-test was performed to investigate which of the top 100 genes associated with survival had significantly different expression levels between the NLR-low group and

NLR-high group among all measured genes by Cox proportional-hazards models using the likelihood ratio test statistic.

All tests were 2-sided with a significance level of 0.05. False discovery rate (FDR) multiple testing was used to adjust the p values. FDR P -value <0.15 was considered statistically significant. SAS 9.4 software (SAS Institute, Cary, NC, USA) was used to perform all analyses.

Results

Seventy-seven eligible patients were enrolled in the present study (Supplementary Figure 1). The baseline characteristics of the patients are summarized in Supplementary Table 1. Forty-four (57%) of the patients were men, the median age of the patients was 63 years, and 82% had left-sided tumors. In the patient cohort, the ORR was 71.8%. The median PFS and OS were 11.2 months (95% CI 9.2–14.5 months) and 36.2 months (95% CI 26.7–not reached), respectively. Consequently, a total of 71 patients who passed the internal Quality Control metrics and had available data on NLR were included in this analysis.

NLR and patient characteristics

The median NLR in the 71 patients was 2.68 (range, 0.78–9.95). The patient characteristics were evaluated according to NLR status (i.e., low or high according to the cut-off value of 2.68). The distribution of NLR low or high did not differ between left-sided tumors and right-sided tumors ($P=0.31$). *BRAF* mutations were detected more frequently in patients with NLR-high tumors (18.8% vs. 2.9%, $P=0.048$) (Supplementary Table 2).

Association of NLR with clinical outcomes

An analysis using Spearman's rank correlation coefficient indicated that the NLR correlated with PFS ($r_s=-0.24$, $P=0.040$) as well as with OS ($r_s=-0.29$, $P=0.011$) in the entire cohort (Supplementary Figure 2). In the study cohort excluding patients who were censored from the analysis of PFS and OS, the NLR was associated with OS ($r_s=-0.53$, $P=0.0024$), but not with PFS ($r_s=-0.20$, $P=0.14$). When the patients were stratified according to the median value of the NLR, patients with NLR-low ($n=36$) had significantly longer PFS than those with NLR-high ($n=35$) (median 11.8 months vs. 9.1 months, hazard ratio [HR] 0.57; 95% CI 0.33–0.97; $P=0.036$). The median OS in the patients with NLR-low was significantly longer than that in the patients with NLR-high (median 42.8 months vs. 26.7 months; HR 0.45; 95% CI 0.22–0.94; $P=0.029$) (Figure 1). The ORR was slightly, but not significantly higher in the NLR-low group than in the NLR-high group (75.0% vs. 68.6%; $P=0.60$) (Table 1). The NLR-low group had significantly better outcomes than the NLR-high group in terms of PFS and OS in a multivariable analysis adjusted for ECOG PS, the number of organs involved, and tumor location (HR 0.55; 95% CI 0.31–0.95; $P=0.033$ and HR 0.44; 95% CI 0.20–0.96; $P=0.038$, respectively). On the other hand, there was no significant association of outcomes with ECOG PS, the number of organs involved, or tumor location in the multivariable analysis (Table 2).

We performed an exploratory analysis to investigate the association between the NLR and clinical outcomes according to primary tumor sidedness. The frequency of NLR-low was

33% in the right-sided tumor group and 53% in the left-sided tumor group. Patients with NLR-low had slightly, but not significantly better clinical outcomes in terms of PFS and OS regardless of tumor sidedness. Both PFS and OS were numerically longer in NLR-low patients than in NLR-high patients, even among patients with right-sided tumors (Supplementary Table 3).

Immune-related genes associated with NLR

First, we checked immune-related genes that were associated with survival in 71 patients by Cox proportional-hazards models using the likelihood ratio test statistic. Then, two-sample t-tests were performed to investigate which of the top 100 genes had significantly different expression levels between the NLR-low and NLR-high groups (Supplementary Table 4). The two-sample t-test revealed that the expression levels of the *LYZ*, *TYMP*, and *CD68* genes differed significantly between the 2 groups (t-test *P*-value <0.005, FDR *P*-value <0.15) (Table 3). The expression levels of all 3 genes were higher in patients with NLR-high than in patients with NLR-low.

Discussion

NLR showed a significant relationship with OS in patients with mCRC treated with 1st-line cetuximab-based chemotherapy. Our data demonstrated that NLR-low was significantly associated with favorable outcomes as compared with NLR-high when the cut-off value was the median. In addition, this is the first report showing that the expression levels of the *LYZ*, *TYMP*, and *CD68* genes differed significantly between the NLR-low and NLR-high groups. All of the 3 genes had positive correlation with the NLR.

A few studies suggested that the NLR might serve as a predictor of the response to bevacizumab-based chemotherapy in mCRC;¹⁶ however, it is not currently used for treatment decision-making. An Australian multicenter study is on-going to evaluate the relations between the host inflammatory response as measured by the NLR and treatment outcomes in patients with mCRC who received bevacizumab-based 1st- and 2nd-line treatment (ClinicalTrials.gov: NCT01588990). On the other hand, a recent retrospective study investigating the role of NLR in patients who received 1st-line cetuximab treatment for mCRC showed that elevated NLR was an independent predictor of shorter PFS and OS in 95 patients extracted from the clinical database of a single institution.¹⁷ Our results were consistent with those of the previous study, but we could demonstrate that the NLR was significantly associated with the clinical outcomes of cetuximab treatment using data from prospective multicenter trials. The NLR may serve as a prognostic marker in patients with CRC treated with chemotherapy regardless of targeted drugs.

In several studies, the NLR independently predicted survival in patients with mCRC treated with chemotherapy followed by resection or chemotherapy when a cut-off value of 5 was used to designate NLR-high or NLR-low.^{8,18} A few studies showed that a preoperative NLR of greater than 3 correlated with overall and cancer-specific survival in CRC patients with resectable tumors.^{19,20} In a post hoc analysis of the MRC COIN trial, a high derived NLR was defined using a cut-off value of 2.22.²¹ On the other hand, Kubo et al. have indicated the impact of the preoperative NLR on long-time survival in patients with CRC using the

median value as a cut-off.²² In our study, we used the median value, 2.68, to divide patients into the NLR-high or low groups. Accordingly, we performed an exploratory analysis of our cohort using a cut-off value of 5.0 since the value has been used in several previous studies. The patients with high-NLR had a shorter survival time than those with low-NLR (median PFS, 5.8 vs. 11.3 months; median OS, 27.2 vs. 42.8 months); however, the differences were not statistically significant. The cut-off value used to divide patients into the high- or low-NLR groups may differ among patient cohorts and depend on patient characteristics or tumor status.

The antitumor activity of cetuximab may be affected by extracellular immune mechanisms. We have reported that immune-related genes are associated with clinical outcomes in mCRC patients treated with cetuximab.²³ Therefore, immune-related genes in the tumor microenvironment may contribute to NLR status which are associated with survival of cetuximab treatment. We could identify 3 genes, *LYZ*, *TYMP*, and *CD68*, which contributed to the level of the NLR. *TYMP* and *CD68* are both expressed in macrophages, particularly tumor-associated macrophages (TAMs). In the tumor microenvironment, *TYMP* stimulates tumor growth by promoting angiogenesis and evasion of apoptosis.²⁴ Moreover, it has been reported that *TYMP* was expressed in tumor-infiltrating macrophages, being associated with tumor angiogenesis and poor prognosis in patients with intestinal-type gastric cancer.²⁵ Gene expression levels of lysozyme (*LYZ*) and signal transducer and activator of transcription 1 (*STAT1*) genes are associated with a macrophage signature.²⁶ *LYZ* expression levels also positively correlate with the numbers of CD68+pSTAT1+ and CD68+CMAF- macrophages. A recent study demonstrated that the combined use of a macrophage marker including CD68 with pSTAT1 or CMAF can identify TAMs which play a significant role in tumor development.²⁷ Accumulation of the TAMs in the tumor is associated with a high NLR. We investigated number of immune-related genes including cytokine genes using a multi-gene panel. A high NLR has been shown to correlate with a distinct cytokine profile including pro-inflammatory cytokines and angiogenic cytokines;³ however, we found the 3 macrophage-related genes as significant ones for the NLR status, leading that NLR status might depend on macrophage-related genes rather than cytokine-related genes. To the best of our knowledge, this is the first time to report that genes encoding for activities on TAMs may affect the NLR related with the outcomes of cetuximab combination chemotherapy.

In this study, the number of registered samples was relatively small. It is difficult to exactly assess the association between the NLR and clinical outcomes in patients with mCRC treated with chemotherapy. However, the results of our study were consistent with those of previous studies. It would be of great interest to investigate whether the NLR can serve as a predictor of the response to chemotherapy in patients with mCRC, potentially providing clinicians with a useful hint on to improve treatment in CRC. In our study, it was not possible to evaluate the NLR as a predictive marker for the response to cetuximab treatment since the patient cohort comprised only patients who received cetuximab combination chemotherapy.

Conclusion

The NLR is significantly associated with survival time in mCRC patients treated with 1st-line chemotherapy with cetuximab. Genes encoding for activities on macrophages may affect the NLR.

Clinical Practice Points

- Few clinical studies have investigated the association between NLR and treatment with cetuximab-based chemotherapy in mCRC.
- The NLR may reflect immune cells modulating specific cytokine signals in the tumor microenvironment; however, which immune-related genes affect the NLR remain unclear.
- In 77 patients with *KRAS* exon2 wild-type mCRC from prospective trials of 1st-line chemotherapy with cetuximab, the association between the NLR and clinical outcomes was evaluated using Spearman's rank correlation coefficient. Also, expression levels of 354 immune-related genes were measured in tissue samples by the *HTG EdgeSeq Oncology Biomarker Panel*.
- The Spearman's rank correlation coefficient showed that the NLR was associated with PFS and OS. When stratified by the median value of the NLR, both PFS and OS were significantly different between the NLR-low and NLR-high groups.
- The expression levels of the *LYZ*, *TYMP*, and *CD68* genes differed significantly between the NLR-low and NLR-high groups. Genes encoding for activities on macrophages may affect the NLR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank the patients, their families, and the investigators who participated in the JACCRO CC-05 and CC-06 trials. We also thank Toshifusa Nakajima for study support, Peter Star (Medical Network K.K.) for English editorial support, John Luecke, Debrah Thompson, and Bonnie LaFleur (HTG Molecular Diagnostics, Inc., Tucson, United States) for gene expression analysis, and Atsushi Kakimoto and Nahoko Hirabayashi (SRL, Inc., Tokyo, Japan) for genetic testing.

Funding

This work was supported by the Japan Clinical Cancer Research Organization (JACCRO), and partly supported by the National Cancer Institute under Grant number P30CA014089; the Gloria Borges WunderGlo Foundation-The Wunder Project; Dhont Family Foundation; San Pedro Peninsula Cancer Guild; Daniel Butler Research Fund; and Call to Cure Fund.

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Figure 1.
Kaplan-Meier curves of clinical outcomes according to the median value of neutrophil-lymphocyte ratio. A) progression-free survival, B) overall survival

Table 1.

Clinical outcomes according to the neutrophil-lymphocyte ratio status

outcomes	NLR >2.68 (n=35)	NLR ≤2.68 (n=36)	P-value
CR, % (n)	2.9 (1)	5.6 (2)	
PR, % (n)	65.7 (23)	69.4 (25)	
SD, % (n)	20.0 (7)	19.4 (7)	
PD, % (n)	5.7 (2)	0 (0)	
NE, % (n)	5.7 (2)	5.6 (2)	
ORR, % (n)	68.6 (24)	75.0 (27)	0.60 [*]
Median PFS, month	9.1	11.8	0.036 ^{**}
Median OS, month	26.7	42.8	0.029 ^{**}

^{*} P-value was estimated by Fisher's exact test

^{**} P-value was estimated by log-rank test

NLR, neutrophil-lymphocyte ratio; ORR, objective response rate; PFS, progression-free survival; OS, overall survival

Table 2.

Multivariable analysis for progression-free survival and overall survival

	PFS			OS		
	HR	95%CI	P-value	HR	95%CI	P-value
ECOG PS						
0 vs. 1	0.79	0.19 – 3.36	0.75	0.29	0.064 – 1.28	0.10
No. of organs involved						
1 vs. 2	0.86	0.49 – 1.52	0.60	0.47	0.20 – 1.08	0.074
Tumor location						
Left vs. right	0.60	0.28 – 1.29	0.19	0.41	0.16 – 1.04	0.061
NLR						
Low vs. high	0.55	0.31 – 0.95	0.033	0.44	0.20 – 0.96	0.038

NLR, neutrophil-lymphocyte ratio; PFS, progression-free survival; OS, overall survival

Table 3.

Significant genes ($P < 0.05$) expressed significantly differently between NLR-high (N=32) and -low (N=35) groups

Genes	T-test P -value	FDR P -value
<i>LYZ</i>	0.001	0.104
<i>TYMP</i>	0.004	0.145
<i>CD68</i>	0.004	0.145
<i>IFNGR1</i>	0.008	0.190
<i>IR4R</i>	0.023	0.467
<i>STAT1</i>	0.031	0.490
<i>TNFRSF1B</i>	0.037	0.490