Abstract: Toll-like receptor (TLR) 9 plays a role in intestinal inflammation that, in turn, is related to the tumorigenesis of colorectal cancer. Nuclear factor kB (NFkB), and interferon regulatory factor (IRF) 5 and IRF7 can be activated by TLR9 and induce the production of proinflammatory cytokines and type I interferon, respectively. This study investigated the mRNA expressions of TLR9 and its downstream signaling molecules in both the tumor and the normal tissues of colorectal cancer.

Eighty-four subjects with colorectal cancer were consecutively recruited at a community-based hospital, and the mRNA expression of TLR9, NFkB, IRF5, IRF7, interleukin 6 (IL6), and interferon α/β/γ receptor 1 (IFNAR1) in the tumor and normal tissue were determined by real-time reverse transcription polymerase chain reaction using TaqMan FAM-labeled MGB probes (Life Technologies, Carlsbad, CA).

The tumor had higher percentages of detection of TLR9, IFNAR1, and IL6 mRNA expressions than normal tissue. The absence of detectable TLR9 mRNA expression was associated with an absence of significance between IL6 and NFkB or IRF5, but not that between IRF7 and IFNAR1 in both the tumor and the normal tissues. An absence of detectable IFNAR1 mRNA expression in the tumor (hazard ratio: 3.77; 95% confidence interval: 1.22–11.60) and advanced stage (stages III and IV, 7.86; 1.76–35.40) were significant predictors for overall survival.

IFNAR1 is a predictor for overall survival and mRNA expression is correlated to IRF7, but not TLR9 in colorectal cancer. The results cast doubt on the usefulness of TLR9 agonist in treating colorectal cancer.

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INTRODUCTION

Toll-like receptors (TLRs) are major transmembrane-signaling pattern-recognition receptors and play essential roles in innate immunity. The main functions of TLRs are induction of inflammation and establishment of adaptive immunity, and their signaling can induce robust production of inflammatory cytokines or type I interferons. Tumorigenesis can be affected in many aspects by inflammation and TLR stimulation. TLRs can either participate in immune reaction against cancer cells or promote tumorigenesis by enhancing chronic inflammatory process. Several TLRs agonists have been developed to treat cancers based on their immunomodulatory effects. However, the possible anti-cancer effects of activated TLRs may be offset by tumor-expressing TLRs and their signaling.

The activation of TLR9 by unmethylated cytosine-phosphate-guanine (CpG) dinucleotides can initiate a cascade of innate and adaptive immune responses that include cell-mediated Type 1 helper T cell (Th1) and humoral immune reactions. The TLR9 signaling pathway can induce the production of inflammatory cytokines through nuclear factor kB (NFkB) and interferon regulatory factor (IRF5), and type I interferon through IRF7. Although TLR9 is mainly expressed on B cells, mature macrophages, monocytes, and plasmacytoid dendritic cells (pDCs), its expression has been found on tumor cells of lung cancer, hepatocellular carcinoma, ovarian tumors, prostate cancer, and esophageal cancer. TLRs expressed on tumor cells can promote growth, contribute resistance to apoptosis, and increase invasiveness. The findings that TLR9 expressions are decreased in hyperplastic and villous polypos from patients who develop colorectal cancer suggest a possible protective role for TLR9 expression against malignant transformation in the colorectal mucosa. TLR9 plays a role in the tumorigenesis of colorectal cancer.

Half of the TLR-targeting drugs under development aim at TLR9 because of easy synthesis and strong induction of Th1 immune response. Although the application of TLR9 agonists in the treatment of cancer is quite promising, CpG 7909 failed to show effects in treating non-small cell lung cancer. The complex interactions among immune cells, tumor cells, and their microenvironment may render the anti-cancer effects of TLR agonists unpredictable. Numerous studies of TLR9 agonists used with colorectal cancer xenografts have shown their effects in decreasing tumor size, prolonging survival, and preventing lung metastasis. Clinical trials using TLR9 agonists against colorectal...
cancer are underway. It would be interesting to know the expression of TLR9 and molecules of its related pathway in colorectal cancer. This study use real-time reverse transcription polymerase chain reaction (RT-PCR) to determine the mRNA expressions of TLR9, NFKB, IRF5, IRF7, interferon α/β/ω receptor 1 (IFNAR1), and interleukin 6 (IL6) in the tumor and normal tissues of subjects with colorectal cancer.

MATERIALS AND METHODS

The Keelung branch of Chang Gung Memorial Hospital is a community-based hospital located in the north pole of Taiwan and responds for the diagnosis or treatment of around 75% (184 out of 247 in 201120) of the total subjects with colorectal cancer in the Keelung city. Eighty-four subjects with a preoperative diagnosis of colorectal cancer were consecutively recruited at our institution from September 2011 to January 2013 after the informed consent had been signed. Histopathologic evaluation was performed based on the diagnostic criteria of the World Health Organization, and all of the tumors were diagnosed as adenocarcinoma. The staging assessment was carried out according to the American Joint Committee on Cancer TNM-classification (7th edition). Clinical and pathologic characteristics were reviewed and recorded. The tumor size was defined as the product of longitudinal and horizontal dimensions. The mean follow-up time was 17.2 ± 8.5 months with 21 deaths observed. The work was approved by the Institutional Review Board of Chang Gung Memorial Hospital.

Real-Time Polymerase Chain Reaction

The tumor and normal colorectal tissue (>10 cm away from the margin of tumor) were embedded in OCT compound (Tissue-Tek, Sakura Finetek USA, Inc, Torrance, CA) within 30 minutes of surgical resection and stored at −20°C for <2 weeks before RNA extraction. A 2 mm³ portion of frozen tumor or normal colorectal tissue was minced, and total RNA was extracted with the RNeasy Mini kit (Qiagen, Hilden, Germany) from the margin of tumor) were included in the OCT compound and treated with RQ1 RNase-Free DNase (Promega, Madison, WI) at 37°C for 10 minutes. The purity of RNA was determined spectrophotometrically and the optical density (OD)260/OD280 values of all the samples were >1.8. The complementary DNA (cDNA) was generated by reverse transcription (SuperScript III, Invitrogen, Carlsbad, CA) using 1 μg of total RNA. PCR amplification of glyceraldehyde-3-phosphate dehydrogenase was done to confirm the integrity of cDNA. Real-time RT-PCR was performed on a BioRad iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) with the following primers and TaqMan FAM-labeled MGB probes (Life Technologies, Carlsbad, CA): Hs99999903_m1 for β-actin; Hs00152973_m1 for TLR9; Hs01014809_g1 for IRF7; Hs09985639_m1 for IL-6; and Hs00158114_m1 for IRF5. For each 0.5-mL Eppendorf tube, 12.5 μL primer and probe mix, 9.25 μL RNase-free water, and 2 μL of cDNA were added to reach a total volume of 25 μL. The cycling conditions for real-time PCR were the following: preincubated with uracil-N-glycosylase at 50°C for 2 minutes, AmpliTaq Gold activation at 95°C for 10 minutes followed by 60 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute. The sizes of amplicons from real-time PCR were checked by gel electrophoresis for their deviation from those provided by the manufacturer. Duplicated cDNA samples of both the tumor and the normal tissues from the same patient and the no-template control for each gene were included in the same real-time PCR experiment. Baseline and threshold values were automatically determined and the expression level of gene was evaluated through normalized gene expression [delta-delta cycle threshold (ddCt)] by the BioRad iQ5 optical system software (version 2.1). The normalized expression of a target gene was estimated by

\[
\text{RFU}_{\text{target}} + \text{RFU}_{\text{β-actin}} + \text{Mean(NTC}_{\text{target}}+\text{β-actin})
\]

where RFU is the relative fluorescence units and NTC_target + β-actin is the sum of RFU of no-template controls for the target gene and β-actin. The real-time PCR test for each sample was duplicated and the reported data was the average of 2 readings. No discordance of mRNA detectability for any gene was observed between the duplicates.

Statistical Analysis

Data analyses were done by SPSS version 18 (SPSS, Inc., Chicago, IL) and R. The differences in categorical variables were detected using χ² or Fisher exact test. Mann–Whitney U or Kruskal–Wallis test was used to test the differences of continuous variables between or among groups. McNemar test was used to compare the distribution of 2 related dichotomous variables. The correlations between dichotomous and continuous variables were presented as ϕ and Spearman correlation coefficients, respectively. The Cochran–Armitage test provided by Mstat software (version 6.1.2, downloaded from http://www.mcgarle.wisc.edu/mstat/) was used to test a trend in the proportion of detectable mRNA expression against pathological or clinical features. The Cox proportional hazards regression model with stepwise selection by Akaikie information criterion (AIC) was used to determine the significance of the following: gender, age, carcinoembryonic antigen (CEA), C-reactive protein (CRP), advanced stage (stages III and IV), and the absence of detectable mRNA expression of individual molecule in tumor or normal tissue as predictors for overall survival (an example of R code: stepAIC(coxph(Surv(fuDays, Death) ~ gender + age + gender + CEA + CRP + advStage + dIFNAR1, data = x)) for the detectability of IFNAR1 mRNA). A P value <0.05 was considered statistically significant.

RESULTS

The characteristics of 84 subjects with colorectal cancer are shown in Table 1. The tumor had higher percentages of detectable mRNA expression in TLR9, IFNAR1, and IL6 than normal tissue (Table 2). The proportion of subjects with detectable TLR9 expression in the normal tissue increased significantly (Cochran–Armitage trend test, P = 0.04) with histological grade: 8/18 in low, 29/56 in moderate, and 9/10 in high grade. Subjects with detectable IL6 mRNA in the tumor or normal tissue had higher serum CEA levels than those without (median 4.48 (0.5–1326) vs 2.14 (0.5–908) with P = 0.048 in the tumor, and 5.04 (0.5–1326) vs 2.14 (0.5–908) with P = 0.008 in the normal tissue). The distributions of age, sex, tumor stages, distant metastasis, tumor size, and CRP were not different between subjects with or without detectable mRNA expression of all molecules in the tumor or normal tissue.

Positive ϕ correlations of mRNA detectability between molecules present in the tumor but not in the normal tissue were as the follows: IRF7 and IFNAR1; TLR9; and IL6 or IRF5. A
positive correlation between the detectability of IRF5 and IRF7 in normal tissue was not seen in the tumor (Figure 1).

Spearman correlation coefficients of mRNA expression between molecules were illustrated in Figure 2. For the tissues with detectable TLR9 mRNA, TLR9 was significantly correlated with all except IFNAR1 in the normal tissue, and the correlations between IFNAR1 and IL6 or IRF5 were not significant in the tumor. For tissue samples without detectable mRNA expression of TLR9, NFkB had significant correlations with all except IL6 in the normal tissue and with none in the tumor. The mRNA expression of IRF7 had significant correlations with that of IFNAR1 with or without the presence of detectable TLR9 mRNA in both the tumor and the normal tissues. IFNAR1 was seen in the tumor but not in the normal tissue. No significant correlation between the detectability of TLR9 and IRF7 was found in both the tumor and the normal tissues.

Cox regression with stepwise selection by AIC found an absence of detectable IFNAR1 mRNA expression in the tumor (hazard ratio: 3.77; 95% confidence interval: 1.22–11.60; P = 0.021), advanced stage (7.86; 1.76–35.40; P = 0.007), and age (1.05; 0.995–1.10; P = 0.074) as a subset of predictors for overall survival.

**TABLE 2. Percentage of Detectable mRNA Expression in Normal and Tumor Tissues**

| Characteristic | Normal tissue, % | Tumor, % | P value |
|----------------|-----------------|---------|--------|
| TLR9           | 54.8            | 73.8    | 0.008  |
| NFkB           | 89.3            | 94.0    | 0.219  |
| IRF7           | 97.6            | 98.8    | 1.0    |
| IRF5           | 73.8            | 75.0    | 1.0    |
| IFNAR1         | 64.3*           | 83.3    | 0.005  |
| IL6            | 64.3*           | 73.8    | 0.021  |

*Significantly different from tumor tissue. IFNAR1 = interferon α/β/ω receptor 1, IL6 = interleukin 6, IRF5 = interferon regulatory factor 5, NFκB = nuclear factor κB, TLR9 = Toll-like receptor 9.

**FIGURE 1.** Phi correlation coefficients of mRNA detectability between molecules of the TLR9/NFκB/IRF5/IL6 (in light gray) and TLR9/IRF7/IFNAR1 (in open rectangle) pathways. A large font-size number denotes a correlation with a P value <0.05. Significant positive correlations between the detectability of TLR9 and IRF5 or IL6, and that of IRF7 and IFNAR1 were seen in the tumor but not in the normal tissue. No significant correlation between the detectability of TLR9 and IRF7 was found in both the tumor and the normal tissues. IFNAR1 = interferon α/β/ω receptor 1, IL = interleukin, IRF = interferon regulatory factor, NFκB = nuclear factor κB, TLR9 = Toll-like receptor.

**DISCUSSION**

Colorectal cancer had higher proportions of detectable TLR9, IFNAR1, and IL6 mRNA expression in the tumor than in the normal tissue. Significant positive correlations between...
the detectability of TLR9 and IL-6 were seen in the tumor but not in the normal tissue. The absence of detectable TLR9 mRNA expression was associated with an absence of significance in the correlation between IL-6 and NFκB or IRF5, but not that between IRF7 and IFNAR1 in both the tumor and the normal tissues. In addition to advanced stage, an absence of detectable IFNAR1 mRNA expression in the tumor was a significant negative predictor for the overall survival of colorectal cancer.

The binding of CpG DNA to TLR9 can induce the production of proinflammatory cytokines through IRF5 and NFκB, and type I interferon through IRF7. TLR9 agonist can activate pDCs to secrete type I interferon and express costimulatory molecules such as CD80 and CD86, with the result of initiating potent cell-mediated Tc1 and humoral B-cell responses. TLR9 agonists for cancer treatment are under active development because of their capability of stimulating a strong Tc1 response, and their easy synthesis and economic benefit. However, TLR signaling acts as a double-edged sword in cancer therapy. Cancer cells can utilize TLR in tissue homeostasis for their growth and survival by producing various immunosuppressive factors, promoting cell growth and angiogenesis, and inducing cytotoxic and antipapoptotic factors. TLR9, similar to other TLRs, is actively expressed by tumor cells in a variety of cancers. A higher proportion of detectable TLR9 mRNA in the tumor than in the normal tissue may lead to a predominance of tumor-promoting effects, if TLR9 agonists are used in colorectal cancer.

IRF5 is needed for the induction of IL6 by the TLR–MyD88 pathway. IRF5 and NFκB p50 are key coregulators of IL6 expression following TLR9-mediated activation of human pDCs. The important role of IRF5 in IL6 expression is suggested by significant correlations between their mRNA detectability and levels in the presence of TLR9 mRNA in both the tumor and the normal tissues. The role of NFκB in IL6 expression is suggested similarly. The importance of TLR9 in the IRF5–IL6 or NFκB–IL6 pathway is suggested by the absence of significant correlations between IL6 and IRF5 or NFκB in both the tumor and the normal tissues in its absence. TLR9 is associated with the NFκB/IRF5/IL6 pathway in colorectal cancer. IL-6 plays important roles in immune responses, cell survival, and proliferation, and the NFκB–IL6–STAT (signal transducer and activator of transcription) 3 pathway links innate immunity to tumorigenesis. IRF5 is antitumorigenic through proapoptotic and cell cycle regulatory effects. NFκB, with its expression found in many cancer cells, can promote or inhibit tumorigenesis. Absence of TLR9, NFκB, IRF5, or IL6 mRNA expression in the tumor or normal tissue was not a significant predictor for overall survival of patients with colorectal cancer. The association of TLR9 mRNA expression with the NFκB/IRF5/IL6 pathway may have uncertain effects on the tumorigenesis and not affect overall survival in colorectal cancer.

IRF7 is the master regulator of type I interferon production and has both oncogenic properties and antitumor effects. IRF7 was downregulated in breast and lung cancer. High expression of IRF7-regulated genes in primary tumors of breast cancer is associated with prolonged bone metastasis-free survival. The binding of interferon to a substantial amount of IFNARs is needed for the expression of tunable interferon-stimulated genes to mount an efficient antiproliferative response. Primary IFNAR1−/− mouse embryonic fibroblasts are tumorigenic and the expression levels of IFNAR1 mRNA are significantly decreased in human gastric cancer tissues. IFNAR1 is one of the candidate tumor suppressor genes deleted in microsatellite stable sporadic colorectal cancer. The absence of detectable IFNARG1 mRNA in tumor tissue was a negative predictor for overall survival of patients with colorectal cancer. The associations between detectable mRNA levels of IRF7 and IFNAR1 were significant with or without the presence of TLR9 mRNA in both the tumor and the normal tissues. IFNAR1 plays an important role in colorectal cancer and its mRNA expression relates to IRF7 but not to TLR9.

High TLR9 expression is associated with a worse histological grade in esophageal cancer. TLRs play a pivotal role in intestinal inflammation that, in turn, may be linked to tumorigenesis. The presence of detectable TLR9 mRNA expression in the normal tissue of colorectal cancer increased with histological grade. A high serum level of IL6 is associated with high serum level of CEA, and IL6 can increase the expression of CEA on the surface of colon cancer cells. The association between IL6 and CEA was suggested by a high serum CEA levels in the presence of detectable IL6 mRNA expression in both the tumor and the normal tissues.

This study has some limitations. First, this study is limited by a small number of patients enrolled in a single institution. However, the study was done with a limited selection bias in a community-based hospital that cares the majority of patients with colorectal cancer in a city of northern Taiwan. Second, this study is observational with most of the statistics based on correlation. Correlation is not causation but it sure is a hint. Third, mRNA rather than protein was determined in this study. Nevertheless, analysis of TLR expression at the mRNA level can provide valuable information. Fourth, real-time PCR was run in duplicate rather than in triplicate and had 60 cycles of denaturation/annealing/extension. However, discordance of mRNA detectability between duplicates were not observed in the studied genes. Significant positive correlations between the detectability of TLR9 and IRF5 or IL6 were seen in the tumor but not in the normal tissue. Furthermore, there was no significant correlation between the detectability of TLR9 and IRF7 in both the tumor and the normal tissues. The results suggest that TLR9 is associated with the IRF5–IL6 pathway but not IRF7 at least in the tumor of colorectal cancer. The analysis of correlations between detectable mRNA levels on the presence or absence of detectable TLR9 mRNA expression also suggests that the mRNA expression of TLR9 is not correlated with those of IRF7 and IFNAR1. Both analyses on the detectability and detectable levels of mRNA similarly suggest that TLR9 is not associated with the IRF7–IFNAR1 pathway.

Type I interferon is mainly regulated by IRF7 and exerts an antiproliferative effect against cancer cells through its binding to IFNARs and activation of the Janus kinase–STAT1/2 pathway in the host cells. A high correlation between IFNAR1 and IRF7 mRNA expression and the absence of detectable IFNAR1 mRNA as a negative predictor for overall survival suggest the important role of the IRF7–IFNAR1 pathway in colorectal cancer. Although TLR9 is an upstream molecule of IRF7, the lack of its association with the IRF7–IFNAR1 pathway casts doubt on the usefulness of TLR9 agonists in treating colorectal cancer.

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