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Expression of Interleukin (IL)-12 mRNA in Gastric Carcinoma Specimens: Cellular Antitumor Immune Responses

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Background and Objectives: Several tumor-related antigen peptides that are recognized by autologous cytolytic T cells (CTL) have been reported. However, most human solid tumors, including gastric carcinoma, are only weakly immunogenic. In this study, we focused on interleukin (IL)-12 and interferon-γ (IFN-γ) as key cytokines for estimating positive cellular immune responses.

Methods: To estimate the immunogenicity of gastric carcinomas, we examined IL-12 and IFN-γ at mRNA levels by reverse transcription-polymerase chain reaction assay (RT-PCR) in tumor specimens and adjacent nontumor specimens from 36 gastric carcinoma patients.

Results: IL-12 expression was detected in 12 tumor specimens and in only two adjacent nontumor specimens ($P = .003$). The frequency of IFN-γ gene expression was higher in the IL-12 mRNA-positive tumor specimens than in the IL-12 mRNA-negative tumor specimens ($P = .015$). In the IL-12 mRNA-positive tumors, IFN-γ expression was higher in the tumor specimens than in the adjacent nontumor specimens ($P = .007$). Conversely, in the IL-12 mRNA-negative tumors, IFN-γ expression was lower in the tumor specimens than in the nontumor specimens ($P = .03$). Many tumor-infiltrating mononuclear cells, predominantly T cells, were found in four of the 12 IL-12-mRNA-positive tumor specimens and in none of the 24 IL-12-mRNA-negative tumor specimens ($P = .008$).

Conclusions: These data suggest that possible immune responses against a tumor may occur at the mRNA level in approximately one-third of gastric carcinomas. *J. Surg. Oncol. 1998;67:11–17.*

KEY WORDS: **interleukin-12 (IL-12); gastric carcinoma; RT-PCR; immune response**

INTRODUCTION

Several tumor-related antigen peptides that are recognized by autologous cytolytic T cells (CTL) have been reported [1,2]. Furthermore, it has been demonstrated that CTL clones can be successfully established from tumor-infiltrating T cells collected from resected melanoma tissues [3,4]. These results indicate that specific cellular immune reactions can be induced at the site of melanomas. However, it is very hard to induce CTL in many other types of solid tumors, including gastric carcinomas. It is currently difficult, if not impossible, reliably to estimate the antigenicity of a tumor.

In mice, it has been demonstrated that exposure of

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TABLE I. PCR Primers and Conditions*

	Primers	PCR conditons	Fragment size
β-actin	5′:GTGGGGCGCCCCAGGCACCA 3′:CTCCTTAATGTCACGCACGATTTC	94°C for 60 sec, 58°C for 60 sec, and 72°C for 120 sec (35 cycles)	541bp
IL-12[a] (p40)	5′:TCACAAAGGAGGCGAGGTTC 3′:TGAACGGCATCCACCATGAC	94°C for 60 sec, 59°C for 60 sec, and 72°C for 120 sec (35 cycles)	378bp
IFN-γ[b]	5′:AGTTATATCTTGGCTTTTCA 3′:ACCGAATAATAAGTCAGCTT	94°C for 60 sec, 59°C for 60 sec, and 72°C for 120 sec (35 cycles)	357bp

*PCR, Polymerase chain reaction.
[a]Interleukin-12.
[b]Interferon-γ.

naive T cells to certain antigens and cytokines causes conversion to either the Th1 or the Th2 phenotype [5,6]. Th1 cells mainly play a critical role in the development of cellular immune response, including possible tumor immunity. Conversion to the Th1 cells is facilitated by interleukin (IL)-12 [7]. IL-12 is a 70-kD heterodimeric cytokine consisting of two covalently linked chains, p35 and p40, both required for the bioactivity of the heterodimer [8,9]. IL-12 is mainly produced by macrophages and B cells when they are stimulated with certain antigens and directly stimulates natural killer (NK) and Th1 cells to produce interferon (IFN)-γ [10,11]. In addition, it has been demonstrated that IL-12 facilitates specific CTL responses [4,12] and that endogenous IL-12 is necessary for the rejection of some types of tumors in a mouse system [13]. Recently, several investigators have found evidence for the possible existence of a similar T-cell phenotype in humans [14].

Thus if tumor-related antigens are processed by antigen-presenting cells (APC) such as macrophages and presented to T cells, the expression and secretion of both IL-12 and IFN-γ may be simultaneously induced at the site of the tumor. To investigate this hypothesis, the expression of both of these cytokines was estimated by reverse transcription-polymerase chain reaction amplification (RT-PCR) and semiquantitative PCR analysis on surgically resected tumor specimens. A quantitative determination of the number of mononuclear cells invading the tumor was also performed microscopically. Our studies suggested that tumor-related cellular immune responses at the molecular level may occur in approximately one-third of gastric carcinomas but less than one-ninth of the cases at the cellular level exhibited significant infiltration by T cells.

MATERIALS AND METHODS

Tumor Specimens

The specimens used in this study were obtained during surgery from 36 patients with gastric carcinoma. Tumor and nontumor samples were collected simultaneously from each patient. Each specimen was divided into two blocks, one minced in RNA lysis buffer on ice for extraction and subsequent analysis by RT-PCR, the other fixed in 10% formalin for histopathologic examination.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Gel Electrophoresis

Total RNA from each specimen was isolated by single-step, guanidium thiocyanate-phenol-chloroform extraction [15]. The specimens were maintained on ice and were minced and manually homogenized in the presence of lysis buffer. The RNA fractions were suspended in diethyl pyrocarbonate- treated water and quantitated by absorbance at 260 nm. RT-PCR was carried out according to the Perkin-Elmer/Cetus protocol for reverse transcription (RT) of RNA and amplification of cDNA. Each RT reaction was carried out with 0.5 μg of RNA per sample. Table I lists the primers used and PCR amplification parameters. Aliquots of the PCR products (7.5 μl) were electrophoresed in a 1.5% agarose gel in Tris acetate EDTA buffer at 100 V for 20 min and then visualized with ethidium bromide staining. A sample was scored as positive if the expected PCR product was visualized by ethidium bromide staining. Therefore, a negative result does not necessarily mean no mRNA expression.

Oligonucleotide Primers

The PCR primers for IL-12 [16], IFN-γ [17], and β-actin [18] are summarized in Table I.

PCR Product Verification by Southern Blot

To verify that the PCR amplification was specific for each mRNA, the PCR products were transferred to nylon membranes and probed with a radiolabeled oligonucleotide complementary to sequences within the region flanked by the primer pairs. The blots were hybridized at 50°C with the probes labeled on their 5′ end with γ-32P (γ-32P-ATP; 7000 Ci/mM; ICN Pharmaceuticals, Costa Mesa, CA) and T4 polynucleotide-kinase (Pharmacia, Upssala, Sweden) for 18 h. The membranes were then washed for 10 min with 2 × SSC and 0.1% SDS, followed by 0.2 × SSC and 0.1% SDS at ambient temperature, and subjected to autoradiography.

TABLE II. Expression of IL-12 and IFN-γ in Tumor and Adjacent Nontumor Specimens

	mRNA expression (positive cases/total cases)	
	IL-12[a]	IFN-γ[b]
Tumor specimens	12/36	23/36
Nontumor specimens	2/36	25/36
P value	.003	.307

[a]Interleukin-12.
[b]Interferon-γ.

Semiquantitative PCR

The cDNA produced by RT was subjected to one-half log serial dilutions and separately amplified with β-actin primers or IFN-γ primers by PCR under the conditions indicated above. To maintain identical RT-PCR conditions, the RT-PCR amplifications for the tumor specimens and adjacent nontumor specimens were simultaneously performed. The PCR products specific for β-actin or IFN-γ were visualized with ethidium bromide and the intensity of the PCR products was determined by densitometry. It was thus possible to compare the relative concentrations of IFN-γ mRNA in each tumor specimen with each adjacent nontumor specimen to an accuracy of approximately one-half log. The increase in the IFN-γ mRNA expression was scored as positive when there was more than a one log higher concentration of IFN-γ mRNA in the tumor specimens compared to the adjacent nontumor specimens.

Immunochemical Staining

The resected tumor specimens were stained with mouse monoclonal antibody (DAKO-UCHT-1; DAKOPATIS, Glostrup, Denmark) against the human CD-3 antigen.

Statistics

Fisher's exact probability test was used for the statistical analyses of mRNA expression of the two cytokines between the tumor specimens and the adjacent nontumor specimens, and between mRNA expression and the clinical pathologic parameters. Mann-Whitney's U test was used for correlation analyses between mRNA expression and histologic stage, and between the mRNA expression and depth of tumor invasion.

RESULTS

mRNA Expression of IL-12 and IFN-γ in Tumor and Nontumor Specimens

The expression of mRNA was compared between the tumor specimens and the adjacent nontumor specimens simultaneously collected from 36 gastric carcinoma patients (Table II). IL-12 mRNA was detected in 12 (33.3%) tumor specimens and in only two (5.6%) nontumor specimens ($P = .003$) (Fig. 1).

Relationship Between IL-12 mRNA Expression and IFN-γ mRNA Expression in Tumor Specimens

IL-12 has been shown to induce the production IFN-γ in NK cells and T-cells. If IL-12 protein is secreted from the tumor, there should be a positive correlation between IL-12 and IFN-γ gene expression in the tumor specimens. IFN-γ mRNA was expressed in 11 of the 12 IL-12-positive tumor specimens and in 12 of the 24 IL-12 RT-PCR negative tumor specimens ($P = .015$) (Table III).

Next, we examined the level of transcription of IFN-γ induced by IL-12 using semiquantitative PCR analysis. The IFN-γ mRNA levels were compared between the tumor specimens and adjacent nontumor specimens. In the 12 IL-12 mRNA-positive cases, seven tumor specimens exhibited a more than twofold amplification of IFN-γ mRNA expression, compared with adjacent nontumor specimens ($P = .007$) (Table III). In the 24 IL-12 mRNA-negative cases, only three tumor specimens showed any significant amplification (more than twofold). However, in the IL-12 mRNA-positive cases, a significant decrease in the IFN-γ mRNA level relative to the adjacent nontumor specimen was found in only two specimens. In the IL-12-negative cases, 13 tumor specimens showed a decrease in IFN-γ gene expression relative to the adjacent nontumor specimens ($P = .034$) (Table III).

Thus a significant correlation between IL-12 mRNA expression and IFN-γ mRNA expression was found in both the frequency and quantity at the tumor site, suggesting that IL-12 protein is secreted in some of the IL-12 mRNA-positive cases.

No significant correlation between IL-12 mRNA expression and IFN-γ mRNA expression was found in the nontumor specimens (Table III).

Relationship Between IL-12 mRNA Expression and Mononuclear Cell Infiltration at the Tumor Site

Zitvogel et al. [19] have demonstrated that intratumor injection of IL-12 producing fibroblasts induced a marked T-cell infiltrate within the tumor. The level of mononuclear cell infiltration into the tumor specimens was examined microscopically and compared between the IL-12 mRNA-positive cases and the IL-12 mRNA-negative cases. If a large number of mononuclear cells occupying more than one-third of the tumor area was seen within the tumor, or if a dense lymphatic ring surrounding the tumor mass was seen, it was scored as being a positive infiltration. UCHT-1 staining revealed that the mononuclear cells invading the tumor consisted predominantly of T cells (Fig. 2). Positive infiltration of T cells was found in 4 of the 12 IL-12 mRNA-positive

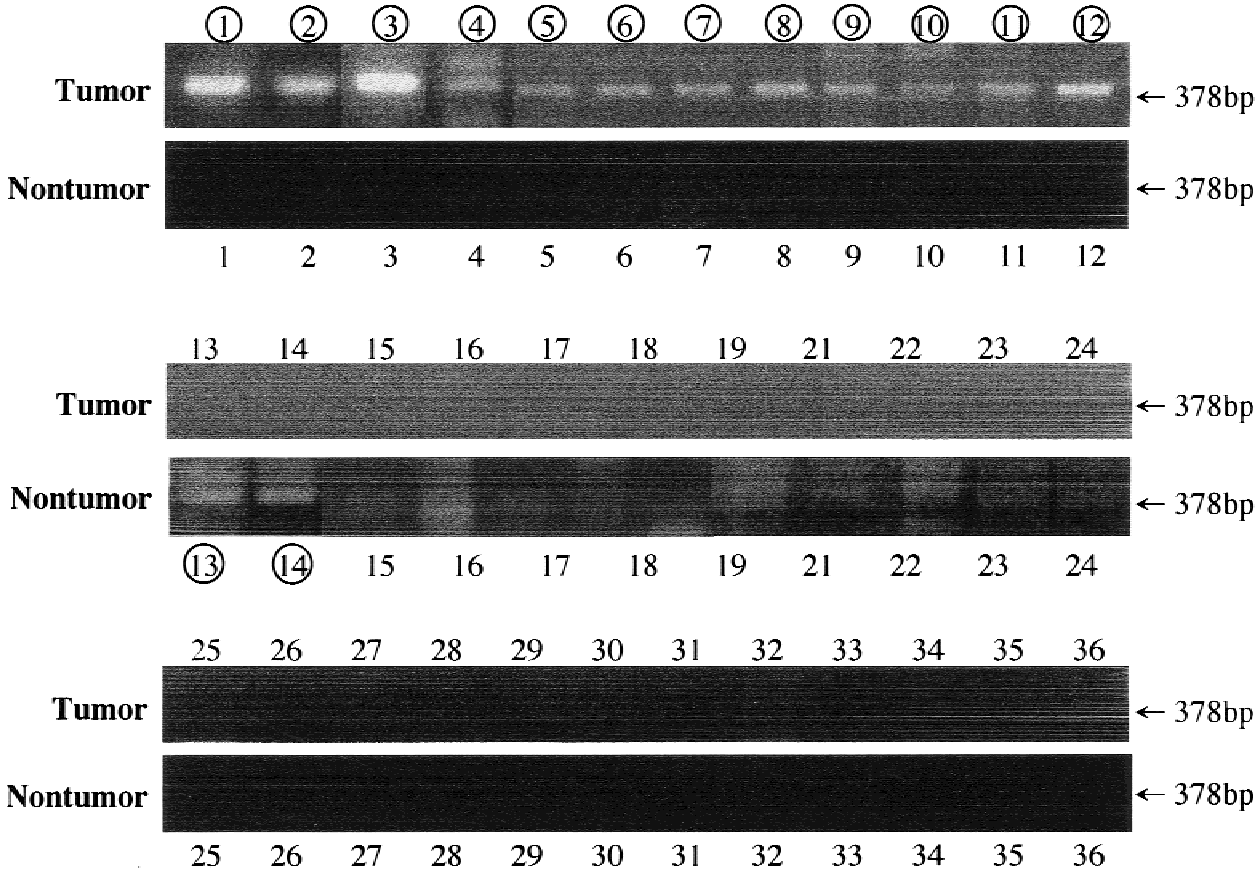

Fig. 1. Polymerase chain reaction (PCR) results for IL-12 expression in all 36 tumor specimens and the adjacent nontumor specimens. The expected 378 bp band is indicated by an arrow. ○ Positive specimen.

specimens and in none of the 24 IL-12 mRNA-negative specimens ($P = .008$) (Table IV).

Relationship Between IL-12 mRNA Expression and Histopathologic Factors From Resected Specimens

Correlation analyses between IL-12 gene expression and several histopathologic factors, including stage, depth of tumor invasion, lumph node metastasis, and histologic type, were performed (Table V).

According to the General Rules for Gastric Cancer Study of the Japanese Research Society for Gastric Cancer, t1, t2, t3, and t4 correspond to tumor invasion of the mucosa or submucosa, muscularis propria, or submucosa, the serosa without invasion of adjacent structures, and adjacent structures, respectively.

When the tumor samples were divided into two groups based upon the degree of differentiation, i.e., a well-differentiated group and a poorly differentiated group, IL-12 gene expression was significantly higher in the poorly differentiated group ($P = .041$). In this study, the poorly differentiated group included moderately differentiated and poorly differentiated adenocarcinomas as well as signet ring cell carcinomas.

DISCUSSION

In this study, we tried to assess the possible presence of tumor-related cellular immune responses at the molecular level in gastric carcinomas. For this assessment, the level of gene expression of two cytokines, IL-12 and IFN-γ, in tumor specimens surgically resected from 36 patients with gastric carcinoma was examined using RT-PCR and semiquantitative PCR analysis. The data presented herein support the hypothesis that natural cellular immune responses against a gastric tumor may occur at the molecular level in approximately one-third of clinical gastric carcinomas.

Since IL-12 is produced by APC stimulated with certain antigens and induces NK and T cells to produce high levels of IFN-γ [10,11] and since IL-12 facilitates specific CTL responses against tumor cells [4,12], the expression of IL-12 and IFN-γ in tumor specimens was assayed by RT-PCR in order to assess the presence of cellular immune responses against the tumor at the tumor site.

Many therapeutic experiments using IL-12 also support the value of IL-12 as an indicator of an active cel-

TABLE III. Relationship Between IL-12 mRNA and IFN-γ mRNA Expression*

	IFN-γ mRNA expression (positive cases/total cases)	Relative levels of IFN-γ mRNA expression (tumor vs nontumor) (no. of cases/total cases)		
		Higher	Same	Lower
Tumor specimens				
IL-12 positive cases	11/12	7/12	3/12	2/12
IL-12 negative cases	12/24	3/24	8/24	13/24
P value	.015	.007	.456	.034
	IFN-γ mRNA expression (positive cases/total cases)	Relative levels of IFN-γ mRNA expression (nontumor vs tumor)[a] (no. of cases/total cases)		
		Higher	Same	Lower
Nontumor specimens				
IL-12 positive cases	2/2	1/2	0/2	1/2
IL-12 negative cases	25/34	14/34	11/34	9/34
P value	.516	.667	.476	.484

*IL-12 = interleukin-12; IFN-γ = Interferon-γ.

[a]Levels of interferon-γ (IFN-γ) mRNA expression in tumor and adjacent nontumor specimens were compared by semiquantitative PCR analysis as described in Materials and Methods. Higher (lower) is defined as a one log or greater increase (decrease) in the IFN-γ mRNA level of the tumor relative to the adjacent nontumor area.

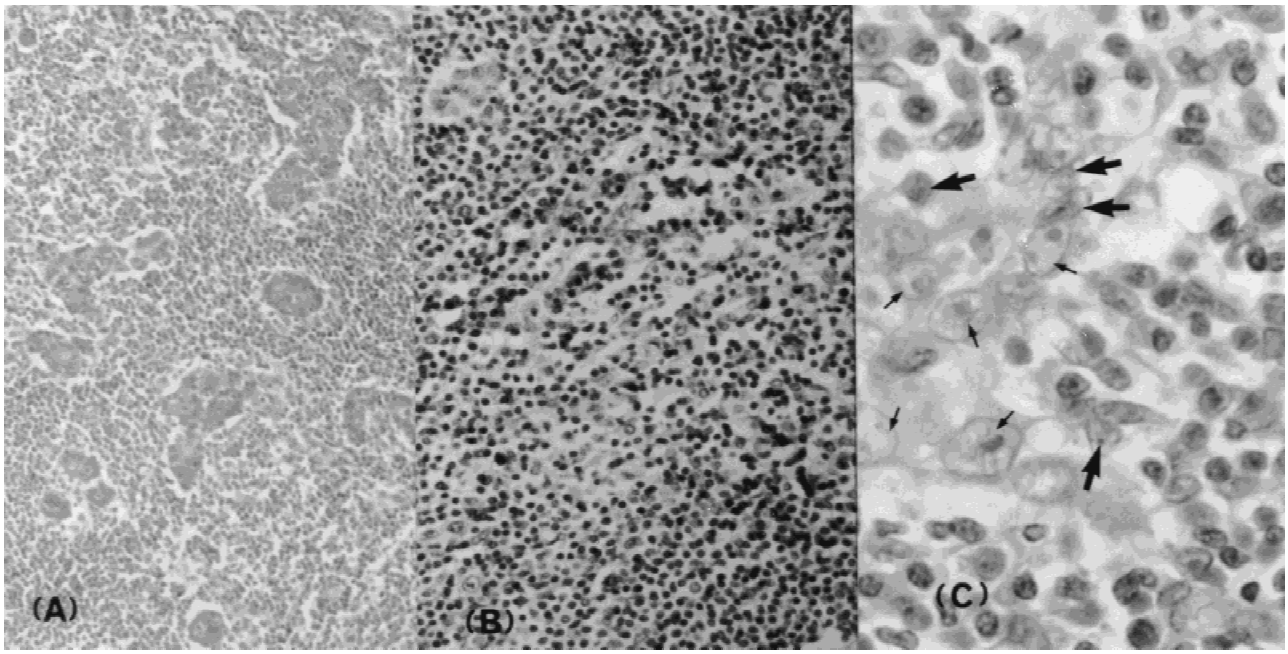

Fig. 2. A representative photomicrograph showing T cell infiltration within a tumor. **A.** Large number of mononuclear cells are seen invading the tumor in foci (H-E staining: 40×). **B.** UCHT-1 staining reveals that the mononuclear cells seen invading the tumor predominantly consist of T cells (UCHT-1 staining: ×40). **C.** Several tumor cell (➡) are smaller than the viable tumor cells (→) and show chromatin condensation, suggesting they are undergoing apoptosis (H-E staining: ×200).

lular immune response in tumors. The therapeutic activity of IL-12 has been observed in various murine tumor models [7,19–21]. The antitumor effect of IL-12 has been shown to involve T cell-mediated events [4,12]. Fallarino et al. [13] have recently demonstrated that endogenous IL-12 is necessary for the rejection of murine mastocytoma P815 variants when it occurs naturally. These observations strongly suggest that endogenous IL-12 may play a critical role in immune responses against tumors. However, it has been generally assumed that the antigenicity of solid tumors is very low, suggesting that very little cytokine is secreted at the tumor site. RT-PCR

TABLE IV. T-Cell-Dominant Inflammatory Infiltrate at the Tumor Site in IL-12-Positive and -Negative Tumor Specimens*

Tumor specimens	T-cell infiltrate (positive cases/total cases)	*P* value
IL-12 positive cases[a]	4/12	
IL-12 negative cases	0/24	.008

*Positive was defined as the presence of a T-cell-predominant mononuclear cell infiltrate occupying more than one-third of the tumor area or a dense T-cell-dominant lymphocytic ring surrounding the tumor mass. In this study, T cells were identified by UCHT-1 staining.
[a]IL-12 = interleukin 12.

TABLE V. Relationship Between IL-12 mRNA Expression and Clinicopathologic Parameters*

	IL-12 mRNA expression (positive cases/total cases)	*P* value
Stage		
I	6/20	
II	0/1	
III	4/9	
IV	2/6	.654[a]
Depth of invasion		
t1	6/18	
t2	1/7	
t3	3/9	
t4	2/2	.388[a]
Nodal states		
node positive	5/14	
node negative	7/22	.544[b]
Histologic type		
well differentiated	0/7	
poorly differentiated[c]	12/29	.041[b]

*IL-12 = interleukin 12.
[a]Mann-Whitney's U-test.
[b]Fisher's exact probability test.
[c]Moderately and poorly differentiated adenocarcinoma and signet ring cell carcinoma.

was used for these assays since it is capable of detecting even low levels of gene expression.

IL-12 mRNA was expressed in 12 patient tumor specimens and in only two of the adjacent nontumor specimens collected simultaneously ($P = .003$) (Table II). This finding indicates that IL-12 gene expression may be relatively specific for sites of tumors or other sites of immune response. Since IL-12 is produced by macrophages, activated B cells, and dendritic cells in response to various microbial agents [11,22,23], this IL-12 gene expression at the tumor site may represent a host response to infection by intracellular pathogens. If so, the risk of bacterial infection may be higher in advanced-stage cases than in early-stage cases. However, no significant difference between advanced-cases and early-cases was found (Table V). No detectable PCR products specific for IL-12 was found in endoscopic biopsy specimens from 10 gastric ulcers (data not shown). This suggests that IL-12 expression was not induced by intracellular pathogens.

IL-12 is an important factor for the induction of IFN-γ-producing T cells in vitro [10,24]. If IL-12 is secreted at the tumor site, IFN-γ mRNA expression also should be enhanced relative to areas without IL-12 secretion. In fact, a higher frequency of tumor specimens compared to adjacent nontumor specimens had increased IFN-γ expression (Table III). Furthermore, the IFN-γ mRNA levels were significantly higher in the IL-12 mRNA-positive tumor specimens than in the adjacent nontumor specimens. Importantly, IFN-γ mRNA levels were lower in the IL-12 mRNA-negative tumor specimens compared with the adjacent nontumor specimens (Table III). It has been demontrated that some tumors secrete immunosuppressive substances such as TGF-β and IL-10 that can interfere with the immunomodulatory functions of IL-12 [25–27]. We found a significant positive correlation between IL-12 and IFN-γ gene expression, suggesting endogenous IL-12 secretion at the tumor site and not consistent with the presence of immunosuppressive substances.

Several experimental reports suggest that the administration of IL-12 at the site of a tumor might stimulate the natural immune response [19,20,28]. Zitvogel et al. [19] have demonstrated that there is an increased number of CD4+ and CD8+ T cells in the lymphocytic ring surrounding tumor nodules in murine tumors injected in situ with IL-12-engineered fibroblasts. These cells eventually invade the tumor in septa and foci. Although the phenotypes of the infiltrating T cells are unclear in our study, similar findings were observed in 4 out of the 12 IL-12 mRNA-positive specimens, but in none of the 24 IL-12 mRNA-negative specimens (Fig. 2, Table IV). In light of these findings, we believe that IL-12-related T cell-mediated immune responses are induced at the cellular level in 1/9 (4/36) of gastric carcinomas.

Kuge et al. [29] have demonstrated that IL-12 augments the generation of autologous tumor-reactive CD8+ cytoxic T cells from tumor-infiltrating lymphocytes (TIL) in breast and renal carcinomas. Their findings and ours suggest that it may be possible to generate tumor-related, T-cell-mediated immune responses even in human tumors already established, that a key cytokine for the induction of this type of immunity is IL-12, and that the secretion level of endogenous IL-12 in established human tumors is too low to induce a visible antitumor effect (Table V). Although it is still unclear if exogenous IL-12 can induce significant T-cell immune responses at the clinical level in gastric carcinomas in which IL-12 mRNA cannot be detected even by RT-PCR, several investigators have demonstrated that endogenous IL-12 was efficacious even in models of spontaneous metastasis and established tumors with low immunogenecity [30,31].

CONCLUSIONS

We analyzed the mRNA expression of IL-12 and IFN-γ at gastric carcinoma sites and adjacent nontumor sites to assess the presence of an active cellular immune response against the tumors. Our studies suggest that IL-12-related cellular immune responses against the tumor may be present in approximately one-thrid of clinical gastric carcinomas.

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