

Role of IL-12p40 in cervical carcinoma

HJMAA Zijlmans¹, S Punt², GJ Fleuren², JB Trimbos³, GG Kenter¹ and A Gorter^{*,2}

¹Department of Gynecology, The Netherlands Cancer Institute Antoni van Leeuwenhoek Hospital, Plesmanlaan 121, 1066CX, Amsterdam, The Netherlands; ²Department of Pathology, Leiden University Medical Center, 2300 RC Leiden, The Netherlands; ³Department of Gynecology, Leiden University Medical Center, 2300 RC Leiden, The Netherlands

BACKGROUND: Previously, we have shown that low *IL-12p40* mRNA expression by cervical cancer cells is associated with a poor survival of cervical cancer patients. As IL-12p40 is both a subcomponent of interleukin (IL)-12 and IL-23, the aim of this study was to elucidate the role of IL-12p40 in cervical cancer.

METHODS: We have measured the expression of *IL-23p19* mRNA, *IL-12p35* mRNA and *IL-12p40* mRNA using mRNA *in situ* hybridisation. The IL-1 and IL-6 were measured by immunohistochemistry.

RESULTS: As IL-23 is a component of the IL-17/IL-23 pathway, a pathway induced by IL-1 and IL-6 in humans, we have studied IL-1 and IL-6 expression. Only a high number of stromal IL-6-positive cells was shown to associate with poor disease-specific survival. The worst disease-specific survival was associated with a subgroup of patients that displayed a high number of IL-6-positive cells and low *IL-12p40* expression ($P < 0.001$). Both a high number of IL-6-positive cells and a high number of IL-6-positive cells, plus low *IL-12p40* expression were shown to be clinicopathological parameters independent of lymph node metastasis, parametrial involvement and Sedlis score ($P = 0.009$ and $P = 0.007$, respectively).

CONCLUSION: Our results with IL-6 and IL-12p40 are in accordance with the hypothesis that the IL-17/IL-23 pathway has a suppressive role in cervical cancer.

British Journal of Cancer (2012) **107**, 1956–1962. doi:10.1038/bjc.2012.488 www.bjcancer.com

Published online 25 October 2012

© 2012 Cancer Research UK

Keywords: cervical cancer; IL-6; IL-12p40

Cervical cancer is a leading cause of morbidity and mortality among women worldwide, especially in the developing countries (Munoz, 2000; Rock *et al*, 2000). Infection with oncogenic types of human papillomavirus (HPV) is an important factor in the development of cervical cancer (Schwartz *et al*, 2001; Waggoner, 2003). The persistent HPV infection induces an inflammatory response. Inflammation is an important component in the majority of tumour types. The outcome of this inflammatory response surrounding the cancer cells is dependent on the composition of the inflammatory infiltrate and locally produced signalling molecules (Coussens and Werb, 2002). Although inflammatory cells within the neoplastic lesion are capable of generating an anti-tumour response, this does not efficiently occur (Elgert *et al*, 1998; Manna and Mohanakumar, 2002).

Inflammatory cells are attracted to the tumour site by locally produced cytokines and chemokines (Balkwill, 2003; Vicari *et al*, 2004). Cervical cancer cells are known to produce an extensive range of cytokines and chemokines, such as CCL2, GM-CSF, TNF α and interleukin (IL)-12 (Clerici *et al*, 1997; Hazelbag *et al*, 2001; Zijlmans *et al*, 2006). In addition to attracting inflammatory cells, these cytokines and chemokines influence the activation status and function of infiltrating antigen-presenting cells and stromal cells, thus influencing the course of the disease (Kusmartsev and Gabrilovich, 2002; Balkwill, 2003).

In a previous study, we have shown that high expression levels or undetectable levels of *IL-12p40* mRNA in cervical carcinoma are associated with an improved overall survival compared with low amounts of *IL-12p40* that were associated with poor survival (Zijlmans *et al*, 2007). As IL-12 is known to stimulate effector cell populations, such as cytotoxic T cells and natural killer cells (Strobl, 2003; Trinchieri, 2003), our results suggest a dual role for *IL-12p40*.

The IL-12 cytokine family includes IL-12, IL-23, IL-27 and IL-35 (Xu *et al*, 2010). From this family, IL-12 and IL-23 share the IL-12p40 subchain. Interleukin-12 is composed of IL-12p40 and IL-12p35, whereas IL-23 is composed of IL-12p40 and IL-23p19. Interleukin-23 has, amongst others, an important role in the IL-17/IL-23 pathway, resulting in the maintenance and expansion of Th17 cells (Korn *et al*, 2009). In addition to IL-23, IL-1 and IL-6 are thought to have an important role in the induction of Th17 cells in humans (Acosta-Rodriguez *et al*, 2007). The effect of IL-23 on cancer progression or cancer eradication is still not clear (Langowski *et al*, 2006; Shan *et al*, 2006).

To further delineate the role of IL-12p40 in cervical carcinoma, we have quantified the mRNA expression levels of *IL-23p19*, and compared its expression level with *IL-12p35* and *IL-12p40* to investigate the relative importance of IL-12 and IL-23 in the tumour microenvironment. In addition, we have investigated the role of IL-1 and IL-6 in the tumour microenvironment by determining both the number of IL-1-positive cells and the number of IL-6-positive cells. Finally, we have assessed the association between *IL-23p19*, *IL-12p35*, *IL-12p40*, high number of IL-6-positive cells and clinicopathological parameters.

*Correspondence: Dr A Gorter; E-mail: A.Gorter@lumc.nl

Received 20 June 2012; revised 3 October 2012; accepted 5 October 2012; published online 25 October 2012

MATERIAL AND METHODS

Patient material

Between 1985 and 1995, 254 untreated patients suffering from primary cervical carcinoma with stage IB and IIA underwent a radical hysterectomy type III with lymphadenectomy. From the tissue obtained, based on the availability of the material, 90 tissue samples were accessible for research. Tissues were routinely embedded in paraffin after 10% formalin fixation. The tissue samples of each patient were examined by a pathologist for the presence of tumour. Tumour percentage varied between 20% and 90%, with median 60%. The characteristics of the patients are depicted in Table 1. Forty-seven patients received post-operative radiotherapy because of either tumour-positive lymph nodes or the presence of positive risk factors described by the Sedlis criteria (Sedlis *et al*, 1999; a combination of two of the following unfavourable prognostic parameters: depth of infiltration ≥ 15 mm (deep stromal invasion; middle or deep third), tumour size ≥ 40 mm and presence of vasoinvasion). Human tissue samples were used according to the guidelines of the Ethical Committee of the Leiden University Medical Centre.

Preparation of IL-23p19, IL-12p35 and IL-12p40 probes

RNA was isolated from frozen human spleen using TRIzol (Invitrogen, Breda, The Netherlands) and first-strand cDNA was synthesised with oligoDT primers and Reverse Transcriptase AMV (both from Roche Diagnostics GmbH, Mannheim, Germany), both according to manufacturer's instructions. Oligonucleotide primers for IL-23-p19, IL-12p35 and IL-12p40 were chosen on the basis of known sequences (see Table 2), and cDNA encoding for the different cytokines was amplified. A pGEM-3Zf(+) Vector (Promega, Madison, WI, USA) was linearised with *Sma*I and the PCR products were cloned into the vector. After transferring the

vector to *E. coli* strain Top 10 (Invitrogen Corp., San Diego, CA, USA), the plasmids were isolated by using the QIAfilter Maxi KITS protocol (QIAGEN GmbH, Hilden, Germany). The sequence of the PCR product was confirmed by DNA sequencing. Plasmids were linearised with *Bam*H1 and *Eco*R1 (both from Boehringer, Mannheim, Germany) in case of IL-12p40, with *Bam*H1 and *Sac*I in case of IL-12p35 and with *Sac*II, *Sal*I and *Spe*I (Boehringer) in case of IL-23p19, using One-Phor-All Buffer Plus (Amersham Biosciences, Roosendaal, The Netherlands). Both strands were translated in a digoxigenin (DIG)-labelled RNA probe according to manufacturer's instructions (Roche Diagnostics GmbH). The concentration of the DIG-labelled sense and antisense RNA probes were determined on a 1% agarose gel stained with ethidium bromide (Sigma, St Louis, MO, USA). Probes were stored at -20°C until further use.

RNA *in situ* hybridisation

The RNA *in situ* hybridisation (RISH) was performed as previously described (de Boer *et al*, 1998; de Boer *et al*, 2000). In short, 3 μm thick paraffin sections were pre-treated and hybridised with 100 ng ml $^{-1}$ DIG-labelled RNA probe diluted in hybridisation mixture containing NaCl and saline-sodium citrate (SSC; Table 2). Hybridisation was allowed for 16 h at either 55 $^{\circ}\text{C}$ (IL-23p19) or 42 $^{\circ}\text{C}$ (IL-12p35 and IL-12p40) in a humidified chamber. Slides were washed 30 min in 2 \times SSC, followed by 45 min in 0.1 \times SSC with 20 mM β -mercaptoethanol (Merck, Darmstadt, Germany), both used at hybridisation temperature (see Table 2). Subsequently, the slides were incubated for 30 min with 2 U ml $^{-1}$ ribonuclease (RNase) T1 (Roche Diagnostics GmbH) in 2 \times SSC, 1 mM EDTA at 37 $^{\circ}\text{C}$. RNA hybrids were detected using, subsequently, mouse anti-DIG (1:2000, Sigma-Aldrich Chemie GmbH, Steinham, Germany), rabbit anti-mouse Ig (1:50, DAKO, Glostrup, Denmark) and mouse alkaline phosphatase anti-alkaline phosphatase (APAAP, DAKO; Hazelbag *et al*, 2001).

Immunohistochemistry

Serial sections, 3- μm thick, of formalin-fixed and paraffin-embedded tissue were mounted on aminopropylethoxysilane-coated slides. Sections were deparaffinised, rehydrated and treated with 0.3% H $_2$ O $_2$ in methanol for 20 min to block endogenous peroxidase activity.

Antigen retrieval was performed (0.01 M citrate, pH 6.0) and sections were rinsed in phosphate-buffered saline (PBS). Subsequently, sections were stained overnight using either a 1:100 dilution of an affinity-purified polyclonal goat anti-human IL-1 β antibody (AF-201-NA; R&D Systems, Minneapolis, MN, USA) or a 1:300 dilution of anti-human polyclonal rabbit anti-IL-6 antibody (Abcam, Cambridge, UK). For anti-IL-1, the slides were incubated with a goat HRP-polymer kit (Biocare Medical, Concord, CA, USA) according to the manufacturer's instructions. For anti-IL-6, the slides were incubated with a biotinylated swine anti-rabbit antibody (1:200; DAKO) and subsequently incubated with a biotinylated horseradish peroxidase - streptavidin complex (1:100, DAKO), respectively. Immune complexes were visualised with diaminobenzidine as previously described (de Boer *et al*, 2000).

(Semi-)quantitative evaluation of RISH and immunohistochemistry

RISH was scored as previously described (Ruiter *et al*, 1998). Intensity was scored as none (0), mild (1), moderate (2) or intense (3) at low magnification ($\times 100$). Furthermore, the percentage of positive tumour cells was determined and divided in 6 groups: 0% (0, absent), 1-5% (1, sporadic), 6-25% (2, local), 26-50% (3, occasional), 51-75% (4, majority) and 76-100% (5, large majority).

Table 1 Summary of clinicopathological features of patients and tumours

Patient and tumour characteristics	Outcome	N ^a
Age	45 (mean) 29-76 (range)	90
FIGO ^b stage	IB IIA	68 21
Lymph node metastasis	No Yes	66 22
Tumour size ^b	< 40 mm ≥ 40 mm	37 26
Depth of infiltration ^b	< 15 mm ≥ 15 mm	58 26
Vascular space involvement ^b	No Yes	39 47
Sedlis criteria ^c	Positive Negative	28 52
Parametrial invasion ^b	No Yes	74 14
HPV status ^d	16, 18 Others	66 15
Histology	Squamous Adenosquamous Adeno Others	58 18 7 6

Abbreviations: FIGO = International Federation of Gynecology and Obstetrics; HPV = human papillomavirus. ^aN is the number of patients/cervical carcinomas. ^bThe number of reported cases is affected by incidental missing cases. ^cSedlis criteria (Sedlis *et al*, 1999): a combination of two of the following unfavourable prognostic parameters: depth of infiltration ≥ 15 mm (deep stromal invasion; middle or deep third), tumour size ≥ 40 mm and presence of vasoinvasion. ^dOnly data for cervical carcinoma samples with a determined HPV type were included. Other subtypes included HPV31 ($n=2$), HPV33 ($n=6$), HPV35 ($n=1$), HPV45 ($n=3$), HPV58 ($n=1$), HPV59 ($n=2$) and HPV68 ($n=1$).

Table 2 RNA probes used and RNA-*in situ* hybridisation conditions

Target	Primer	Sequence (5'–3')	Product (bp)	Accession number	Position	Hybr. temp. (°C)
IL-23p19	Forward	AGAGCCAGCCAGATTTGAGA	487	NM_016584.2	134–620	55
	Reverse	GCAGATTCCTCAAGCCTCAGTC				
IL-12p35	Forward	TGCTCCAGAAGGCCAGACAAAAC	465	XM_003121	320–784	42
	Reverse	CCCGAATTCTGAAAGCATGAAAG				
IL-12p40	Forward	GGACCAGAGCAGTGAGGTCTT	373	XM_004011	189–561	42
	Reverse	CTCCTGTGTTGCCCTCTGA				

Abbreviation: Hybr. temp. = hybridisation temperature.

The sum of both the percentage and the staining intensity of the positive cells resulted in an overall score (0 to 2 to 8). The scores were combined into three groups: category 0 (score 0, no expression), category 1 (scores 2, 3, 4 and 5, low expression) and category 2 (scores 6, 7 and 8, high expression). mRNA expression was scored by two independent researchers without knowing the identity and clinical outcome of the patients. The IL-1-positive cells and IL-6-positive cells were quantified in the tumour by counting the number of stained cells per six, randomly selected, high-power field of view (HPF, × 400).

Statistical analysis

Data from immunohistochemistry as well as RISH are given as the mean ± s.d. Statistical analysis was done using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Data were processed by using the χ^2 -test. Kaplan–Meier survival curves were generated to assess differences in disease-free period (defined as the observation time in months from surgery to relapse of the disease (disease-free survival)) or cumulative disease-specific survival (defined as time in months from surgery to death due to cervical cancer). A Cox regression was used for multivariate survival analysis. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Patients

Of the group of 90 patients, 68 patients were diagnosed as FIGO stage IB and 21 patients as FIGO stage IIA, and all underwent radical hysterectomy combined with pelvic lymph adenectomy (Table 1). Forty-seven patients received post-operative radiotherapy because of either tumour-positive lymph nodes or meeting the terms of the Sedlis criteria (Sedlis *et al*, 1999; a combination of two of the following unfavourable prognostic parameters: depth of infiltration ≥ 15 mm, tumour size ≥ 40 mm and presence of vasoinvasion). Twenty-five patients suffered recurrent disease. At the end of the study, 70 patients were alive, 7 suffered from a recurrence and 18 patients had died of disease.

Expression of IL-23p19, IL-12p35 and IL-12p40 in cervical cancer

As IL-12p40 is both a subunit of IL-12 and IL-23, we have determined the expression of IL-23p19. Both IL-23p19 and IL-12p40 were expressed by cervical tumour cells (Figure 1A and C). The expression of IL-12p40 was stronger than the expression of IL-23p19. IL-23p19 was expressed in 63% of the samples ($n = 54$), IL-12p40 was expressed in 54% of the samples ($n = 90$) and IL-12p35 was expressed in 84% of the samples ($n = 90$; Table 3). All samples that expressed either IL-23p19 or IL-12p40 also expressed IL-12p35 mRNA. In contrast, 13 out of 44 samples that expressed IL-12p40 did not express IL-23p19.

A positive correlation between IL-23p19 and IL-12p40 was found ($n = 54$, $r^2 = 0.117$, $P = 0.011$; data not shown). No statistically significant correlation between IL-23p19 and IL-12p35 was found ($n = 54$, $r^2 = 0.061$, $P = 0.072$; data not shown).

Association between IL-23p19, IL-12p35 and IL-12p40, and disease-specific survival in cervical cancer

To investigate the relationship between the expression of IL-23p19, IL-12p35 and IL-12p40, and disease-specific survival, Kaplan–Meier plots were created. A log-rank test was used to determine statistical differences in disease-specific survival. As the absence of IL-12p40 will result in neither IL-12 nor IL-23, we first confirmed that expression of IL-12p40 was associated with poor disease-specific survival (Figure 2A; $n = 48$, log-rank test 5.753, $P = 0.017$) in this cohort. The expression of IL-12p35 (Figure 2B; $n = 74$, log-rank test 0.2019, $P = 0.653$) and the expression of IL-23p19 (Figure 2C; $n = 33$, log-rank test 1.930, $P = 0.165$) were both not significantly associated with disease-specific survival. The expression of IL-23p19, IL-12p35 or IL-12p40 showed no significant difference in disease-free survival (data not shown).

Presence of IL-6-positive cells and association with disease-specific survival in cervical cancer

The presence of IL-23p19 suggests that IL-23 may sustain a Th17-positive cell population in cervical cancer. As differentiation towards the IL-17/IL-23 pathway is thought to occur in the presence of IL-1 and IL-6 in humans (Acosta-Rodriguez *et al*, 2007), we have determined the presence of IL-1 and IL-6-positive cells, using immunohistochemistry. Interleukin-1 was predominantly expressed by cells in the stromal compartment. Occasionally, tumour cells also showed weak IL-1 expression. No statistically significant association between low or high number of IL-1-expressing cells and disease-specific survival was observed (Figure 3A). Interleukin-6 was expressed by both cells in the epithelial (tumour cell) compartment and cells in the stromal compartment (Figure 1E). No significant association was observed between low or high IL-6 expression of cells in the epithelial compartment and disease-specific survival (Figure 1F). Subsequently, we quantified the number of IL-6-positive cells in the stroma. The presence of a high number of IL-6 stromal positive cells (median 17 IL-6-positive cells/HPF) significantly associated with disease-specific survival (Figure 3B; $n = 83$, log-rank test 12.57, $P < 0.001$). No statistical significant difference was observed for disease-free survival (data not shown). We also determined whether disease-specific survival was associated with the presence of both a high number of IL-6-positive stromal cells and low IL-12p40. In this latter case, an even stronger decrease in disease-specific survival was observed (Figure 3C; $n = 47$, log-rank test 20.38, $P < 0.001$).

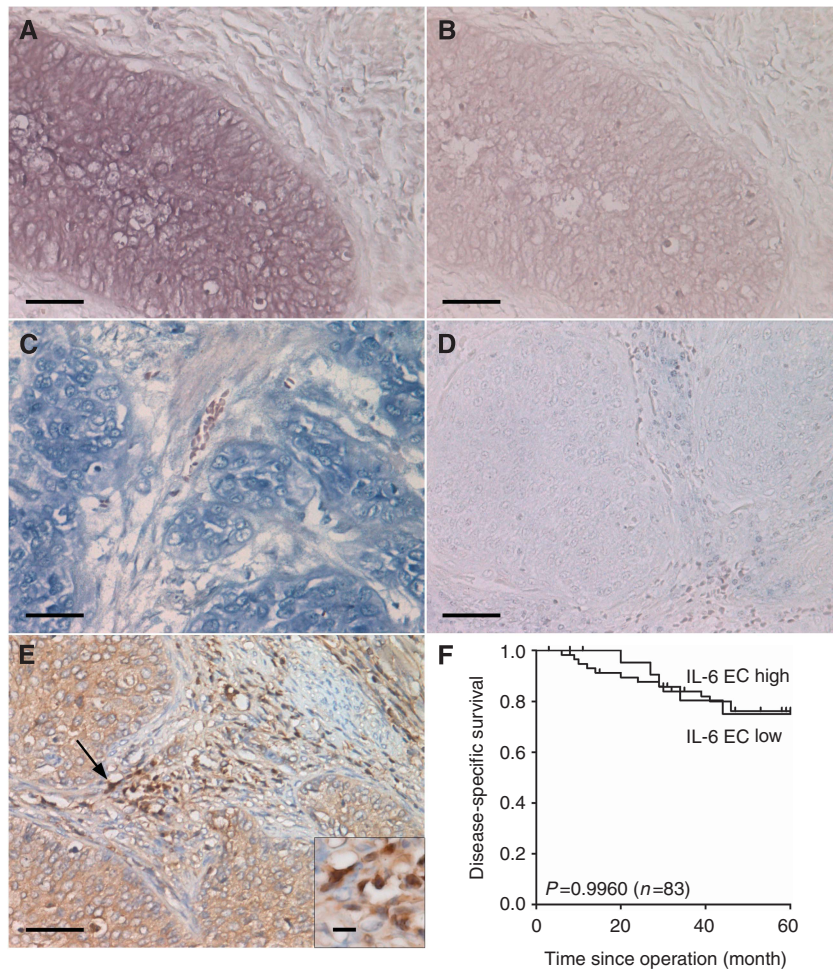


Figure 1 The expression of *IL-23p19*, *IL-12p40* and IL-6. The expression of *IL-23p19* and *IL-12p40* were determined using RISH, and the expression of IL-6 was determined using immunohistochemistry as described in the Materials and Methods ($\times 250$ magnification). (**A**) Cervical tumour, *IL-23p19* RISH. Tumour cells stain positive (moderate) for *IL-23p19*; (**B**) negative (sense) control of *IL-23p19* RISH; (**C**) cervical tumour, *IL-12p40* RISH. Tumour cells stain positive (strong) for *IL-12p40*; (**D**) negative (sense) control of *IL-12p40* RISH. (**E**) IL-6 staining of cervical cancer tissue. Both cells in the epithelial compartment (EC) as well as cells in the stroma express IL-6. Arrow indicates positive stromal cells. Detail ($\times 400$ magnification) of IL-6-positive cells in the stroma; and (**F**) association between cells in the epithelial compartment with low (IL-6 EC low) and high IL-6 (IL-6 EC high) expression and disease-specific survival. Bars correspond to $50\ \mu\text{m}$ in **A–E** and to $10\ \mu\text{m}$ in the inset of **E**. No significant association between low or high IL-6 expression of the epithelial cells with disease-specific survival was observed.

Association between low *IL-12p40* expression, high number of IL-6-positive stromal cells and clinicopathological parameters

To determine the relevance of our findings, we associated our immunological findings with clinicopathological parameters. First, a univariate Cox analysis was performed, using the clinical parameters, Sedlis criteria (two out of three of the following criteria positive: tumour size ≥ 40 mm, vasoinvasion and deep stromal invasion), lymph node metastasis and parametrial involvement and the immunological parameters, low *IL-12p40* expression, high number of IL-6-positive cells and high number of IL-6-positive cells, plus low *IL-12p40* expression. In the univariate Cox analyses, all the included parameters showed a significantly increased hazard ratio (HR; Table 4). Subsequently, a multivariate Cox analysis with the three clinicopathological parameters and each of the significantly immunological parameters was performed. In this case, two of the three immunological parameters, high number of IL-6-positive stromal cells ($P = 0.009$; HR, 7.447) and high number of IL-6-positive stromal cells and low *IL-12p40* expression ($P = 0.007$; HR, 20.123) were shown to be independent predictors of poor disease-specific survival.

DISCUSSION

In a previous study, we found an association between low expression of *IL-12p40* and poor disease-specific survival, whereas high expression of *IL-12p40* or lack of expression of *IL-12p40* were associated with a favourable disease-specific survival (Zijlmans *et al*, 2007). As *IL-12p40* combines with both *IL-12p35* and *IL-23p19*, to form *IL-12* and *IL-23*, respectively, in the present study, we have further investigated the role of *IL-12p40* in cervical cancer.

Both *IL-23p19* and *IL-12p35* were expressed in the majority of the samples. Out of the 44 samples that expressed *IL-12p40*, 13 samples did not express *IL-23p19*. As *IL-12p35* expression seems to be ubiquitous in cervical cancer (Zijlmans *et al*, 2007), the level of *IL-12p40* or *IL-23p19* expression most probably determines whether *IL-12*, *IL-23* or both are expressed. In our study, we observed a trend ($P = 0.061$; $n = 54$) between *IL-23p19* or *IL-12p35* expression, whereas in the study of Wolf *et al* (2010) in ovarian cancer ($n = 112$) a significant correlation between the expression of *IL-23p19* and *IL-12p35* was found. The discrepancy between our results and the results of Wolf *et al* (2010) may be due to the smaller size of our study group. Very few studies have investigated the association between local expression of *IL-12* or *IL-23*, and

prognosis. Using immunohistochemistry, IL-12 has been associated with improved survival in patients with (advanced) gastric carcinoma (Ye *et al*, 2007; Nagashima *et al*, 2008). In the study of Wolf *et al* (2010), using RT-PCR, both *IL-12p35* and *IL-23p19* were associated with a superior outcome. In a multivariate analysis, *IL-12p35* was found to be an independent factor for overall survival of ovarian carcinoma. As stated previously, we have observed a statistically significant association between low expression of *IL-12p40* and poor disease-specific survival in cervical carcinoma (Zijlmans *et al*, 2007). In the present study and our previous study (Zijlmans *et al*, 2007), we did not find a significant association between either *IL-23p19* or *IL-12p35* expression and disease-specific survival. As *IL-23p19* and *IL-12p35* are both expressed, it is important to determine which cytokine, IL-23 or IL-12, has a dominant effect on the tumour microenvironment.

The molecular interaction between IL-23p19 and IL-12p40 has been studied by Beyer *et al* (2008). These authors reported that the

interface region of IL-23p19 and IL-12p35 on IL-12p40 overlap. Because of different interresidue interactions of IL-12p35 and IL-23p19 with IL-12p40, these molecules interact with a different affinity with IL-12p40. Therefore, the availability of IL-12p40 in combination with the affinity for IL-12p35 and IL-23p19 may result in skewing of the IL-12/IL-23 response. This is supported by experiments performed by Zwiers *et al* (2011). These authors showed that in an experimental animal model, polymorphic variants of IL-12p40 can skew IL-12/IL-23 synthesis (Zwiers *et al*, 2011). Thus, both differences in protein interactions between IL-23p19 and IL-12p35 on the one hand and IL-12p40 on the other hand, and genetic polymorphisms in the protein chains, such as IL-12p40, contribute to the amounts of IL-12 and IL-23 formed. Our results support a previously suggested immunosuppressive role for IL-23 (Langowski *et al*, 2006). This is further supported by a study in ovarian carcinoma where genetic differences in the IL-23 receptor have been reported to influence prognosis (Zhang *et al*, 2010).

In contrast, it has also been shown that overexpression of IL-23 reduces tumour growth and metastasis formation, and that IL-23 is able to elicit a strong cytotoxic T-cell memory response (Lo *et al*, 2003; Shan *et al*, 2006), underscoring our view that the level of expression of the different cytokines and chemokines has an important role in the final outcome.

Our data suggest that in the presence of a limited amount of *IL-12p40* the biological effect of IL-23 dominates, whereas in the presence of a high amount of *IL-12p40* the biological effect of IL-12 prevails. As IL-12 polarises the immune response towards an antiviral response, (Trinchieri, 1994) the favourable cumulative overall survival of patients with a high IL-12-positive cell density (Ye *et al*, 2007; Nagashima *et al*, 2008) and high expression level of *IL-12p35* (Wolf *et al*, 2010) and *IL-12p40* (Zijlmans *et al*, 2007) can be explained by the capacity of this cytokine to increase the lytic activity and the production of interferon- γ of natural killer cells and cytotoxic T lymphocytes (Trinchieri, 1994). Interestingly, we previously observed an association between high expression of *IL-12p40* and high expression of *TGF- β* ($P=0.024$; Zijlmans *et al*, 2007), suggesting that the tumour cells are selected to counter act the effect of IL-12 or skew the response to the IL-17/IL-23 pathway.

In humans, in addition to IL-1, IL-6 and *TGF- β* have been implicated to have a role in the IL-17/IL-23 pathway (Veldhoen *et al*, 2006; Acosta-Rodriguez *et al*, 2007). Previously, we have shown that it is likely that activated *TGF- β* is present in the tumour microenvironment, as PAI-1, a target gene of *TGF- β* , is expressed and expression of PAI-1 is associated with survival (Hazelbag *et al*, 2004). Furthermore, both the integrin $\alpha v \beta 6$ and active matrix metalloproteinase-2, known to activate *TGF- β* , are associated with poor disease-specific survival (Sier *et al*, 2006; Hazelbag *et al*,

Table 3 Correlation between *IL-23p19*, *IL-12p35* and *IL-12p40* expression in cervical carcinoma.

Cytokine	Expression level	<i>IL-12p35</i>			Total n (%) ^a
		Absent	Low	High	
<i>IL-12p40</i>	Absent	14	21	6	41 (46)
	Low	0	14	14	28 (31)
	High	0	3	18	21 (23)
	Total n (%) ^a	14 (16)	38 (42)	38 (42)	90 (100)
P-value				< 0.001	
Cytokine	Expression level	<i>IL-23p19</i>			Total n (%) ^a
		Absent	Low	High	
<i>IL-12p40</i>	Absent	7	1	2	10 (19)
	Low	8	5	11	24 (44)
	High	5	4	11	20 (37)
	Total n (%) ^a	20 (37)	10 (19)	24 (44)	54 (100)
P-value				0.188	
Cytokine	Expression level	<i>IL-23p19</i>			Total n (%) ^a
		Absent	Low	High	
<i>IL-12p35</i>	Absent	1	0	0	1 (2)
	Low	8	5	8	21 (39)
	High	11	5	16	32 (59)
	Total n (%) ^a	20 (37)	10 (19)	24 (44)	54 (100)
P-value				0.619	

^an = number of tumours. The scores were combined into three groups: absent expression, low expression and high expression as described in Materials and Methods. Statistically significant P-value is given in bold.

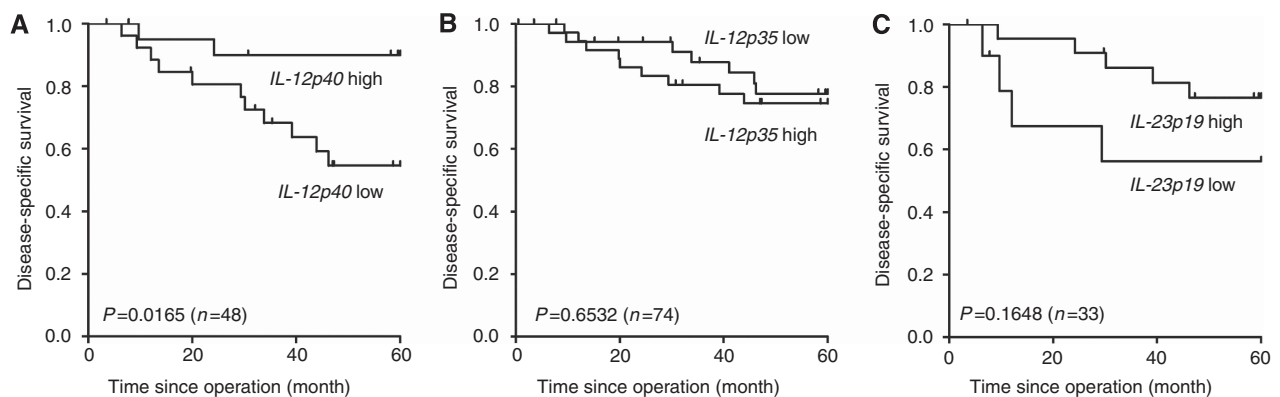


Figure 2 Association between *IL-12p40* (A), *IL-12p35* (B) and *IL-23p19* (C) expression and disease-specific survival in cervical carcinoma. *IL-23p19*, *IL-12p35* and *IL-12p40* were determined by RISH as described in the Materials and Methods.

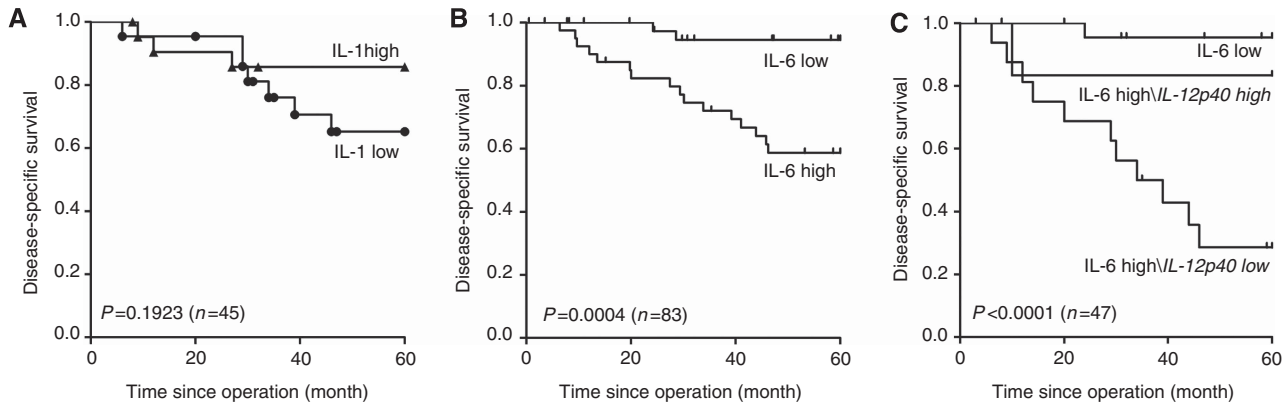


Figure 3 Association between high number of IL-1-positive cells (A), high number of IL-6-positive stromal cells (B) and *IL-12p40* expression in combination with high number of IL-6-positive stromal cells (C) and disease-specific survival. The IL-1 and IL-6 expression were determined by immunohistochemistry as described in Materials and Methods. *IL-12p40* was determined by RISH as described in the Materials and Methods.

Table 4 Cox regression of clinicopathological variables and *IL-12p40* and the number of IL-6-positive cells in cervical carcinoma

Univariate	Hazard ratio	95% CI	P-value
Sedlis positive	4.354	1.608–11.789	0.004
Lymph node metastasis	3.266	1.286–8.296	0.013
Parametrial involvement	3.645	1.411–9.411	0.008
Low <i>IL-12p40</i>	5.231	1.156–23.661	0.032
High number of IL-6 cells	8.975	2.063–39.051	0.003
Low number of IL-6 cells	Reference		
High number of IL-6 cells/low <i>IL-12p40</i>	21.832	2.805–169.915	0.003
<i>Multivariate</i>			
Sedlis positive	3.848	1.270–11.663	0.017
Lymph node metastasis	1.333	0.439–4.052	0.612
Parametrial involvement	1.526	0.542–4.444	0.438
High number of IL-6 cells	7.447	1.659–33.432	0.009
<i>Multivariate</i>			
Sedlis positive	0.706	0.150–3.310	0.659
Lymph node metastasis	2.887	0.836–9.965	0.094
Parametrial involvement	1.168	0.283–4.822	0.830
Low number of IL-6 cells	Reference		
High number of IL-6 cells/low <i>IL-12p40</i>	20.123	2.248–180.147	0.007

Abbreviation: CI = confidence interval. Shown are the log-rank test and P-value of compared expression levels. Statistically significant P-values are given in bold.

2007). Even though the role of TGF- β in inducing Th17 in humans has been questioned, TGF- β may suppress Th1 and Th2 development, thus favoring Th17 development (Santarlaschi *et al*, 2009; Das *et al*, 2009).

In our study, low or high numbers of IL-1-positive cells were not associated with disease-specific survival. The presence of a high number of IL-6-expressing stromal cells was significantly associated with poor disease-specific survival ($P < 0.001$). Previously,

REFERENCES

Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F (2007) Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 8(9): 942–949

IL-6 has been implicated as an autocrine or paracrine growth factor for cervical cancer (Tartour *et al*, 1994; Castrilli *et al*, 1997). Interleukin-6 has been shown to induce VEGF transcription via the STAT3 signalling pathway, thus promoting an angiogenic switch (Wei *et al*, 2003). Indeed, blockade of the IL-6 receptor on cervical cancer cell lines was shown to interfere with cell survival signals and blocked the expression of VEGF (Su *et al*, 2005).

As HPV vaccines will become available for the treatment of metastasised cervical carcinoma, the local cytokine/chemokine profile may be important to discriminate patients with a beneficial immune response from non-responding patients.

In conclusion, *IL-12p40* has at least a dual role in cervical carcinoma by associating with both *IL-23p19* and *IL-12p35*. We have shown that low *IL-12p40* expression was significantly associated with poor disease-specific survival ($P = 0.017$). Also, a high number of stromal IL-6-producing cells was shown to associate with poor disease-specific survival ($P < 0.001$). The worst disease-specific survival was observed in a subgroup of patients that displayed a high number of stromal IL-6-expressing cells and low *IL-12p40* expression ($P < 0.001$). Furthermore, both a high number of stromal IL-6-expressing cells and a high number of stromal IL-6, plus *IL-12p40* expression, were shown to be independent clinicopathological parameters compared with lymph node metastasis, parametrial involvement and Sedlis score ($P = 0.009$ and $P = 0.022$, respectively). Our results with IL-6 and *IL-12p40* are in accordance with the hypothesis that the IL-17/IL-23 pathway has a suppressive role in cervical cancer.

ACKNOWLEDGEMENTS

We thank Enno J Dreef, Natalja T ter Haar, Sandra M Kolkman-Uljee, Nils I Wijtzes and Michel C Verboom for their technical assistance in the Department of Pathology.

Conflict of interest

The authors declare no conflict of interest.

- interleukin-23 and its complex with a high-affinity neutralizing antibody. *J Mol Biol* 382(4): 942–955
- Castrilli G, Tatone D, Diodoro MG, Rosini S, Piantelli M, Musiani P (1997) Interleukin 1alpha and interleukin 6 promote the *in vitro* growth of both normal and neoplastic human cervical epithelial cells. *Br J Cancer* 75(6): 855–859
- Clerici M, Merola M, Ferrario E, Trabattoni D, Villa ML, Stefanon B, Venzon DJ, Shearer GM, De Palo G, Clerici E (1997) Cytokine production patterns in cervical intraepithelial neoplasia: association with human papillomavirus infection. *J Natl Cancer Inst* 89(3): 245–250
- Coussens LM, Werb Z (2002) Inflammation and cancer. *Nature* 420(6917): 860–867
- Das J, Ren G, Zhang L, Roberts AI, Zhao X, Bothwell AL, Van KL, Shi Y, Das G (2009) Transforming growth factor beta is dispensable for the molecular orchestration of Th17 cell differentiation. *J Exp Med* 206(11): 2407–2416
- de Boer WI, Sont JK, van Schadewijk A, Stolk J, van Krieken JH, Hiemstra PS (2000) Monocyte chemoattractant protein 1, interleukin 8, and chronic airways inflammation in COPD. *J Pathol* 190(5): 619–626
- de Boer WI, van Schadewijk A, Sont JK, Sharma HS, Stolk J, Hiemstra PS, van Krieken JH (1998) Transforming growth factor beta1 and recruitment of macrophages and mast cells in airways in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 158(6): 1951–1957
- Elgert KD, Alleva DG, Mullins DW (1998) Tumor-induced immune dysfunction: the macrophage connection. *J Leukoc Biol* 64(3): 275–290
- Hazelbag S, Fleuren GJ, Baelde JJ, Schuuring E, Kenter GG, Gorter A (2001) Cytokine profile of cervical cancer cells. *Gynecol Oncol* 83(2): 235–243
- Hazelbag S, Kenter G, Gorter A, Dreef E, Koopman L, Violette S, Weinreb P, Fleuren G (2007) Overexpression of the alphavbeta6 integrin in cervical squamous cell carcinoma is a prognostic factor for decreased survival. *J Pathol* 212(3): 316–324
- Hazelbag S, Kenter GG, Gorter A, Fleuren GJ (2004) Prognostic relevance of TGF-beta1 and PAI-1 in cervical cancer. *Int J Cancer* 112(6): 1020–1028
- Korn T, Bettelli E, Oukka M, Kuchroo VK (2009) IL-17 and Th17 Cells. *Annu Rev Immunol* 27: 485–517
- Kusmartsev S, Gabrilovich DI (2002) Immature myeloid cells and cancer-associated immune suppression. *Cancer Immunol Immunother* 51(6): 293–298
- Langowski JL, Zhang X, Wu L, Mattson JD, Chen T, Smith K, Basham B, McClanahan T, Kastelein RA, Oft M (2006) IL-23 promotes tumour incidence and growth. *Nature* 442(7101): 461–465
- Lo CH, Lee SC, Wu PY, Pan WY, Su J, Cheng CW, Roffler SR, Chiang BL, Lee CN, Wu CW, Tao MH (2003) Antitumor and antimetastatic activity of IL-23. *J Immunol* 171(2): 600–607
- Manna PP, Mohanakumar T (2002) Human dendritic cell mediated cytotoxicity against breast carcinoma cells *in vitro*. *J Leukoc Biol* 72(2): 312–320
- Munoz N (2000) Human papillomavirus and cancer: the epidemiological evidence. *J Clin Virol* 19(1–2): 1–5
- Nagashima N, Nakayama Y, Inoue Y, Nagata J, Matsumoto K, Minagawa N, Katsuki T, Shibao K, Hirata K, Sako T, Hamada T, Nagata N (2008) Prognostic significance of the local expression of interleukin-12 in patients with advanced gastric cancer. *Anticancer Res* 28(2B): 1277–1283
- Rock CL, Michael CW, Reynolds RK, Ruffin MT (2000) Prevention of cervix cancer. *Crit Rev Oncol Hematol* 33(3): 169–185
- Ruiter DJ, Ferrier CM, van Muijen GN, Henzen-Logmans SC, Kennedy S, Kramer MD, Nielsen BS, Schmitt M (1998) Quality control of immunohistochemical evaluation of tumour-associated plasminogen activators and related components. European BIOMED-1 Concerted Action on Clinical Relevance of Proteases in Tumour Invasion and Metastasis. *Eur J Cancer* 34(9): 1334–1340
- Santarlasci V, Maggi L, Capone M, Frosali F, Querci V, De PR, Liotta F, Cosmi L, Maggi E, Romagnani S, Annunziato F (2009) TGF-beta indirectly favors the development of human Th17 cells by inhibiting Th1 cells. *Eur J Immunol* 39(1): 207–215
- Schwartz SM, Daling JR, Shera KA, Madeleine MM, McKnight B, Galloway DA, Porter PL, McDougall JK (2001) Human papillomavirus and prognosis of invasive cervical cancer: a population-based study. *J Clin Oncol* 19(7): 1906–1915
- Sedlis A, Bundy BN, Rotman MZ, Lentz SS, Mudderspach LI, Zaino RJ (1999) A randomized trial of pelvic radiation therapy vs no further therapy in selected patients with stage IB carcinoma of the cervix after radical hysterectomy and pelvic lymphadenectomy: A Gynecologic Oncology Group Study. *Gynecol Oncol* 73(2): 177–183
- Shan BE, Hao JS, Li QX, Tagawa M (2006) Antitumor activity and immune enhancement of murine interleukin-23 expressed in murine colon carcinoma cells. *Cell Mol Immunol* 3(1): 47–52
- Sier CF, Zuidwijk K, Zijlmans HJ, Hanemaaijer R, Mulder-Stapel AA, Prins FA, Dreef EJ, Kenter GG, Fleuren GJ, Gorter A (2006) EMMPRIN-induced MMP-2 activation cascade in human cervical squamous cell carcinoma. *Int J Cancer* 118(12): 2991–2998
- Strobl H (2003) Molecular mechanisms of dendritic cell sublineage development from human hematopoietic progenitor/stem cells. *Int Arch Allergy Immunol* 131(2): 73–79
- Su JL, Lai KP, Chen CA, Yang CY, Chen PS, Chang CC, Chou CH, Hu CL, Kuo ML, Hsieh CY, Wei LH (2005) A novel peptide specifically binding to interleukin-6 receptor (gp80) inhibits angiogenesis and tumor growth. *Cancer Res* 65(11): 4827–4835
- Tartour E, Gey A, Sastre-Garau X, Pannetier C, Mosseri V, Kourilsky P, Fridman WH (1994) Analysis of interleukin 6 gene expression in cervical neoplasia using a quantitative polymerase chain reaction assay: evidence for enhanced interleukin 6 gene expression in invasive carcinoma. *Cancer Res* 54(23): 6243–6248
- Trinchieri G (1994) Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. *Blood* 84(12): 4008–4027
- Trinchieri G (2003) Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 3(2): 133–146
- Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B (2006) TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24(2): 179–189
- Vicari AP, Treilleux I, Lebecque S (2004) Regulation of the trafficking of tumour-infiltrating dendritic cells by chemokines. *Semin Cancer Biol* 14(3): 161–169
- Waggoner SE (2003) Cervical cancer. *Lancet* 361(9376): 2217–2225
- Wei LH, Kuo ML, Chen CA, Chou CH, Lai KB, Lee CN, Hsieh CY (2003) Interleukin-6 promotes cervical tumor growth by VEGF-dependent angiogenesis via a STAT3 pathway. *Oncogene* 22(10): 1517–1527
- Wolf AM, Rumpold H, Reimer D, Marth C, Zeimet AG, Wolf D (2010) High IL-12 p35 and IL-23 p19 mRNA expression is associated with superior outcome in ovarian cancer. *Gynecol Oncol* 118(3): 244–250
- Xu M, Mizoguchi I, Morishima N, Chiba Y, Mizoguchi J, Yoshimoto T (2010) Regulation of antitumor immune responses by the IL-12 family cytokines, IL-12, IL-23, and IL-27. *Clin Dev Immunol* 2010: 832454
- Ye ZB, Ma T, Li H, Jin XL, Xu HM (2007) Expression and significance of intratumoral interleukin-12 and interleukin-18 in human gastric carcinoma. *World J Gastroenterol* 13(11): 1747–1751
- Zhang Z, Zhou B, Zhang J, Chen Y, Lai T, Yan L, Liang A, Li Y, Wang Y, Chen Y, Zhang L, Xi MR (2010) Association of interleukin-23 receptor gene polymorphisms with risk of ovarian cancer. *Cancer Genet Cytogenet* 196(2): 146–152
- Zijlmans HJ, Fleuren GJ, Baelde HJ, Eilers PH, Kenter GG, Gorter A (2006) The absence of CCL2 expression in cervical carcinoma is associated with increased survival and loss of heterozygosity at 17q11.2. *J Pathol* 208(4): 507–517
- Zijlmans HJ, Fleuren GJ, Baelde HJ, Eilers PH, Kenter GG, Gorter A (2007) Role of tumor-derived proinflammatory cytokines GM-CSF, TNF-alpha, and IL-12 in the migration and differentiation of antigen-presenting cells in cervical carcinoma. *Cancer* 109(3): 556–565
- Zwiers A, Fuss IJ, Seegers D, Konijn T, Garcia-Vallejo JJ, Samsom JN, Strober W, Kraal G, Bouma G (2011) A polymorphism in the coding region of Il12b promotes IL-12p70 and IL-23 heterodimer formation. *J Immunol* 186(6): 3572–3580

This work is published under the standard license to publish agreement. After 12 months the work will become freely available and the license terms will switch to a Creative Commons Attribution-NonCommercial-Share Alike 3.0 Unported License.