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Role of IL-12p40 in cervical carcinoma

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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.

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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, TNFα 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.

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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 $\geqslant$15 mm (deep stromal invasion; middle or deep third), tumour size $\geqslant$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 $-20\,^{\circ}$C until further use.

Table 1 Summary of clinicopathological features of patients and tumours

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

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

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 °C (*IL-23p19*) or 42 °C (*IL-12p35* and *IL-12p40*) in a humidified chamber. Slides were washed 30 min in 2 × SSC, followed by 45 min in 0.1 × 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 × SSC, 1 mM EDTA at 37 °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_2O_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-1β 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 (× 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).

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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	GCAGATTCCAAGCCTCAGTC				
IL-12p35	Forward	TGCTCCAGAAGGCCAGACAAAC	465	XM_003121	320–784	42
	Reverse	CCCGAATTCTGAAAGCATGAAG				
IL-12p40	Forward	GGACCAGAGCAGTGAGGTCTT	373	XM_004011	189–561	42
	Reverse	CTCCTTGTTGTCCCCTCTGA				

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 or 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, $\times 400$).

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, $P<0.001$).

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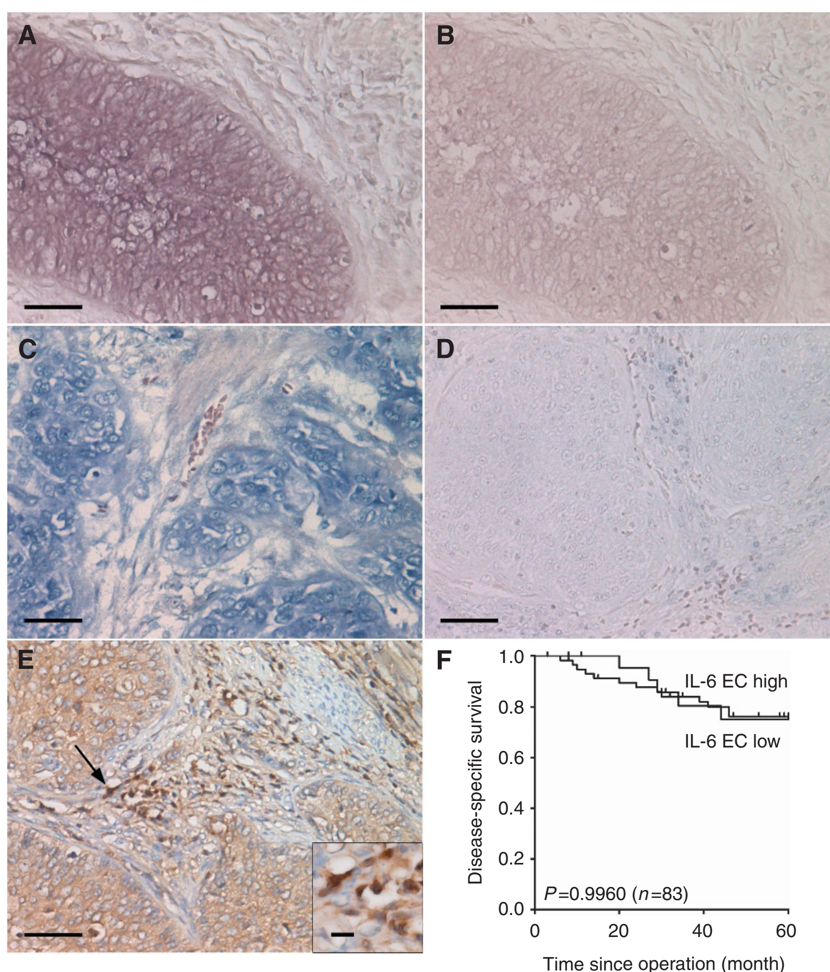

Figure 1 The expression of *IL-23p19*, *IL-12p40* and IL-6. The expression of *IL-23p19* and *IL-12p40* were determined using RISH, and the expression of IL-6 was determined using immunohistochemistry as described in the Materials and Methods (× 250 magnification). (**A**) Cervical tumour, *IL-23p19* 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 (× 400 magnification) of IL-6-positive cells in the stroma; and (**F**) association between cells in the epithelial compartment wit low (IL-6 EC low) and high IL-6 (IL-6 EC high) expression and disease-specific survival. Bars correspond to 50 μm in **A**–**E** and to 10 μm 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-12-p35* 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

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prognosis. Using immmunohistochemistry, 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-23-p19* 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-23-p19 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 import 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 avβ6 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	Absent	Low	High	Total n (%)[a]
		IL-12p35			
IL-12p40	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**
		IL-23p19			
IL-12p40	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
		IL-23p19			
IL-12p35	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

[a] *n* = 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.

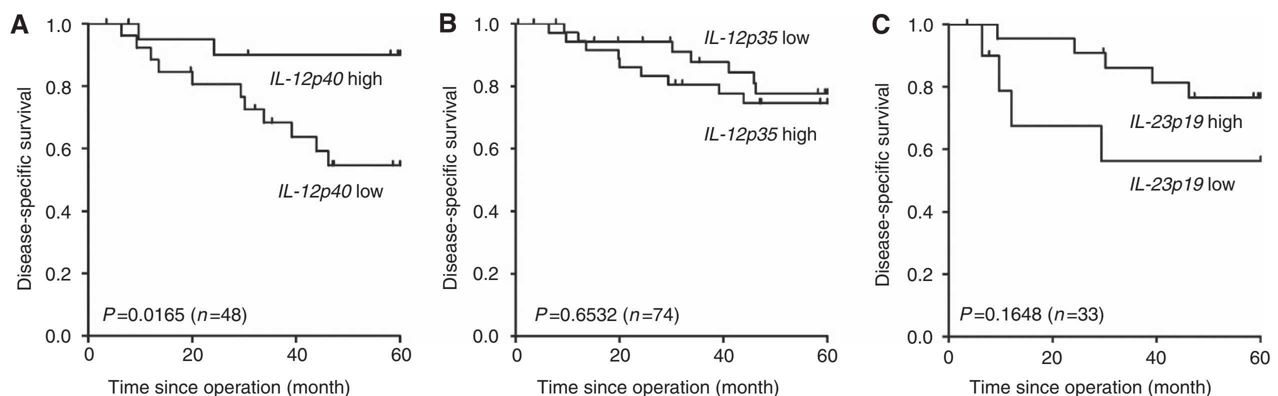

Figure 2 Association between *IL-12p40* (**A**), *IL-12p35* (**B**) and *IL-23p19* (**C**) expression and disease-specific survival in cervical carcinoma. *IL-23p19*, *IL-12p35* and *IL-12p40* were determined by RISH as described in the Materials and Methods.

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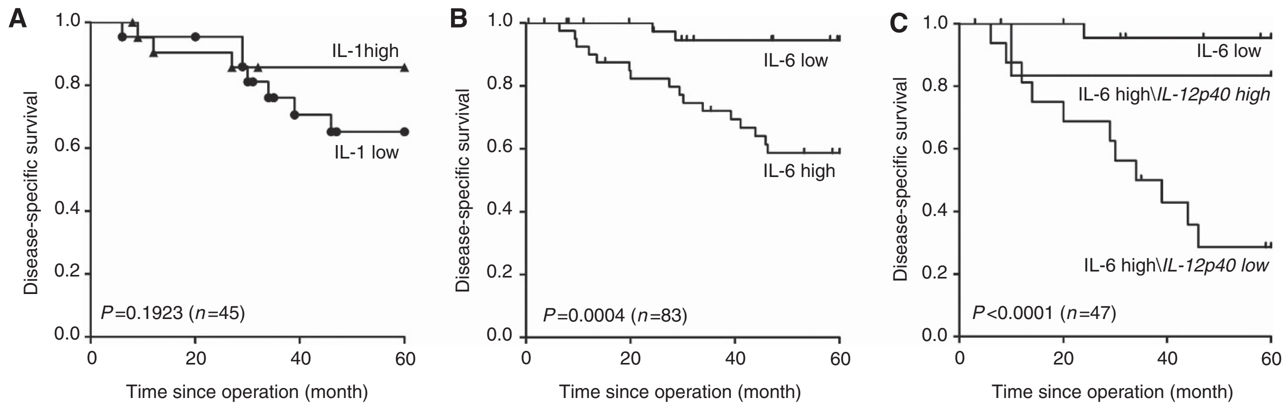

Figure 3 Association between high number of IL-1-positive cells (**A**), high number of IL-6-positive stromal cells (**B**) and *IL-12p40* expression in combination with high number of IL-6- positive stromal cells (**C**) and disease-specific survival. The IL-1 and IL-6 expression were determined by 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 *IL-12p40*	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 *IL-12p40*	21.832	2.805–169.915	**0.003**
Multivariate			
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**
Multivariate			
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 *IL-12p40*	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 (Santarlasci *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, 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.

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Conflict of interest

The authors declare no conflict of interest.

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

Balkwill F (2003) Chemokine biology in cancer. *Semin Immunol* **15**(1): 49–55

Beyer BM, Ingram R, Ramanathan L, Reichert P, Le HV, Madison V, Orth P (2008) Crystal structures of the pro-inflammatory cytokine

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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, Muderspach 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, Mizuguchi 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

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