Marker discovery for response to (chemo)radiation and prognosis in cervical cancer

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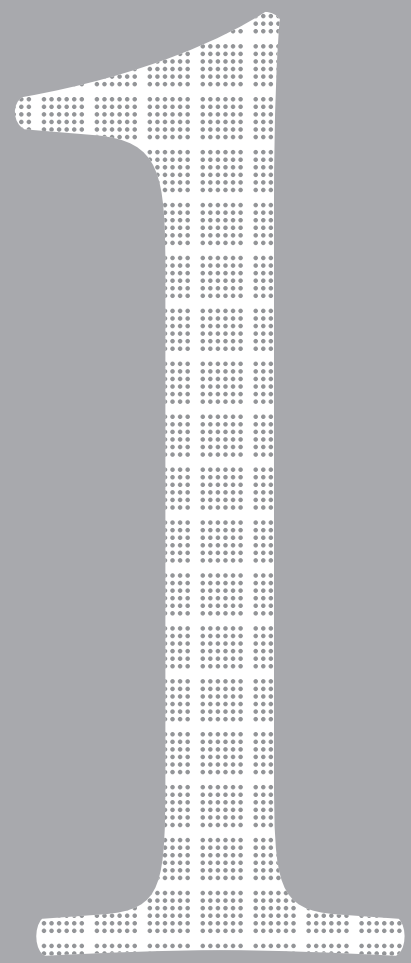
General introduction



General introduction

Cervical cancer is, after breast and colorectal cancer, the third most common cancer among women worldwide, responsible for 529.000 new cases and 275.000 deaths in 2008 (1). The implementation of population-based screening programs for cervical neoplasia in developed countries since the 1960s, has caused a strong reduction in cervical cancer incidence (2), while in developing countries cervical cancer still accounts for 13% of the female malignancies (1). Infection with high-risk human papillomavirus (hr-HPV) has been identified as the most important factor in development of cervical cancer (3). Therefore, improving cervical cancer screening by HPV DNA testing and vaccination against HPV are nowadays of major interest (4,5).

Treatment of early stage cervical cancer patients (FIGO stage Ia-IIa) consists of radical hysterectomy and pelvic lymph node dissection. For this group of patients, presence of pelvic lymph node metastases is the most important prognostic factor (6). Early stage cervical cancer patients with negative lymph nodes have a 5-year survival rate of 90% vs. only 65% in patients with lymph node metastases (7). Patients with lymph node metastases are therefore treated with adjuvant (chemo)radiation. However, the combination of surgery and (chemo)radiation is associated with severe morbidity (8). If presence of metastatic lymph nodes would be known before treatment, primary chemoradiation could be considered, which is equally effective, but associated with a different treatment-related morbidity pattern (8). Currently, no clinicopathological features or cell biological markers are available to predict lymph node status with high sensitivity and specificity. Non- and minimal invasive diagnostic techniques, such as sentinel lymph node biopsy are currently being explored to better identify patients with disease outside the cervix (9).

Standard treatment of locally advanced cervical cancer (FIGO stage Ib2, IIb-IVa) is concurrent cisplatin-based chemoradiation, which has been shown to be more effective than radiotherapy alone (10). Chemoradiation improves both overall and progression-free survival and reduces local as well as distant recurrences (11-13). Response to chemoradiation can be evaluated by gynaecologic examination under general anaesthesia 8 to 10 weeks after completion of treatment. If residual tumor tissue is found, adjuvant radical surgery can be performed (14). Despite improvement in survival of patients with locally advanced cervical cancer, the 5-year overall survival of patients treated primarily with chemoradiation is still only 66% (10). Further improvement of survival rates by intensification of standard chemoradiation is limited, because of resistance to radiotherapy and/or chemotherapy and an increase of short- and long-term side effects (15). Therefore, new treatment modalities are urgently needed to increase the anti-tumor

effect of chemoradiation and thereby improve the survival of cervical cancer patients with advanced stage disease.

At the moment choice of treatment in cervical cancer is generally based on well-known prognostic factors, such as FIGO stage and presence of lymph node metastases. Besides clinicopathological factors, cell biological markers could be of potential clinical relevance. Investigating cell biological markers might lead to identification of potential targets for therapeutic intervention, which in combination with standard treatment may improve survival rates, without causing a major increase in toxicity. Furthermore, cell biological markers could be helpful in predicting presence of pelvic lymph node metastases, response to chemoradiation and prognosis in the individual patient (16).

In this thesis, cell biological markers and pathways associated with lymph node metastases, response to (chemo)radiation and prognosis in cervical cancer are investigated.

Outline of the thesis

Over the last years, many cell biological markers have been studied in relation to survival and/or response to (chemo)radiation in locally advanced cervical cancer. Especially markers involved in tumorigenesis and tumor progression, such as genes associated with apoptosis, angiogenesis and cell growth have been investigated extensively. As the focus of improving survival is nowadays on targeted therapies, knowledge about the predictive and prognostic significance of various markers may help to determine potential targets for therapeutic intervention. The aim of **chapter 2** was to identify prognostic cell biological markers in cervical cancer patients primarily treated with (chemo)radiation and to review their potential application in treatment of advanced stage cervical cancer. A systematic review of well-documented studies on the prognostic and predictive value of cell biological markers comprising $\geq$50 cervical cancer patients, primarily treated by (chemo)radiation was performed.

Several pathways have been identified of which a relation with survival is suggested. Epidermal growth factor receptor (EGFR) is involved in the ErbB signaling pathway, which is often dysregulated in cancer. Autophosphorylation of EGFR leads to activation of two downstream pathways: the Ras/Raf/MEK/ERK pathway and the PI3K/AKT pathway. PTEN (Phosphatase and Tensin Homolog Deleted on Chromosome Ten) acts as a tumor suppressor gene by inhibiting phosphorylation and thereby activation of AKT (17,18). Both downstream EGFR pathways have been shown to be involved in processes associated with carcinogenesis and tumor progression, such as inhibition

of apoptosis, cell migration, cell growth, and angiogenesis (19). However, the prognostic significance of different components of the EGFR pathway in cervical cancer is equivocal, due to small, heterogeneous (frequently a mix of primarily surgically and radiotherapeutically treated) patient populations and methodological differences in immunohistochemistry. Therefore, the aim of **chapter 3** was to determine the relation between expression of proteins involved in the EGFR pathway (EGFR, phosphorylated (p)EGFR, PTEN, pAKT, and pERK) and survival in a well-documented series of early stage cervical cancer patients. For this purpose, immunostaining was performed on tissue microarrays (TMAs) that contain tumor tissue of 336 consecutive early stage (Ib-IIa) cervical cancer patients primarily treated by radical hysterectomy and pelvic lymph node dissection.

Development of metastasis is a complex, multistep process (20). Little is known about biological pathways involved in cervical cancer lymph node metastasis. A better understanding of the molecular mechanism of lymph node metastasis in cervical cancer might contribute to individual treatment strategies. Therefore, in **chapter 4** the aim was to identify cellular tumor pathways associated with pelvic lymph node metastasis in early stage cervical cancer. To identify such pathways, expression array analysis (Affymetrix U133 plus 2.0) was performed on a well defined series of tumor tissues of 20 cervical cancer patients with histologically confirmed pelvic lymph node metastases vs. 19 patients with histologically and clinically confirmed negative lymph nodes. Also individual genes differentially expressed between lymph node positive and negative patients were identified. Potential markers representing the predictive value of pathways were validated in a consecutive series of 274 early stage cervical cancer patients by immunohistochemistry on TMAs.

Tumor resistance to (chemo)radiation can be caused by loss of the ability of tumor cells to go into apoptosis. The extrinsic apoptotic pathway is initiated by activation of death receptors (DRs) expressed on the cell membrane. Apoptosis is triggered by the binding of specific TNF super family ligands, such as binding of tumor necrosis factor–related apoptosis inducing ligand (TRAIL) to its receptors DR4 and DR5 (21,22). Preclinical data indicate a synergistic effect on apoptosis between irradiation and recombinant human (rh)TRAIL in cervical cancer cells (23), making the TRAIL death receptors interesting drug targets. For targeting death receptors, presence of death receptors on cervical cancer cells is required. In **chapter 5** protein expression of DR4, DR5, and TRAIL in cervical cancer was studied and their prognostic and predictive value was determined. Immunostaining of DR4, DR5, and TRAIL was performed on TMAs containing 645 FIGO stage Ia2-IVa cervical cancer patients.

As mentioned previously, the EGFR pathway is involved in processes associated with carcinogenesis and tumor progression, such as inhibition of apoptosis, cell migration, cell growth, and angiogenesis (19). More recently, activation of the EGFR signaling pathway has been reported to induce resistance to (chemo)radiation in cancers (24,25). This indicates that EGFR targeted agents in addition to standard (chemo)radiation might improve treatment efficacy. Therefore, in **chapter 6** the prognostic and predictive significance of EGFR pathway members (EGFR, pEGFR, PTEN, pAKT, and pERK) was studied in 375 consecutive FIGO stage Ib-IVa cervical cancer patients, primarily treated with (chemo)radiation.

During radiotherapy, DNA double strand breaks (DSBs) are introduced to cause cell death. The response to radiotherapy is influenced by proteins that are involved in signaling and repairing DSBs. Ataxia telangiectasia mutated (ATM) is a key protein in DSB signaling (26). In response to DSBs, ATM is activated through auto-phosphorylation and this results in a signaling cascade which leads to DNA-repair (27). Until now, the role of ATM in response to radiotherapy in cervical cancer has only been investigated *in vitro*. These studies showed that ATM downregulation results in radiosensitization of cervical cancer cells (28,29). Protein expression of non-phosphorylated ATM (nATM) and phosphorylated ATM (pATM) has not been studied before in cervical cancer. The aim of **chapter 7** was to examine the role of immunostaining of nATM and pATM in response to (chemo)radiation and survival in the same well-documented series of 375 consecutive cervical cancer patients, primarily treated with (chemo)radiation.

Finally, in **chapter 8** a summary of the results is presented and future perspectives are discussed and **chapter 9** is a summary of the thesis in Dutch.

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Prognostic cell biological markers in cervical cancer patients, primarily treated with (chemo)radiation: a systematic review

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Abstract

The aim of this study was to systematically review prognostic and predictive significance of cell biological markers in cervical cancer patients primarily treated with (chemo)radiation. A PubMed, Embase, and Cochrane literature search was performed. Studies describing a relation between a cell biological marker and survival in $\geq$50 cervical cancer patients primarily treated with (chemo)radiation were selected. Study quality was assessed and studies with a quality score of 4 or lower were excluded. Cell biological markers were clustered on biological function and the prognostic and predictive significance of these markers was described. In total, 42 studies, concerning 82 cell biological markers were included in this systematic review. In addition to cyclooxygenase-2 (COX-2) and serum squamous cell carcinoma antigen levels (SCC-ag), markers associated with poor prognosis were involved in Epidermal Growth Factor Receptor (EGFR) signaling (EGFR and C-erbB-2) and in angiogenesis and hypoxia (carbonic anhydrase 9 and hypoxia-inducible factor-1α). EGFR and C-erbB-2 were also associated with poor response to (chemo)radiation. In conclusion, EGFR signaling is associated with poor prognosis and response to therapy in cervical cancer patients primarily treated with (chemo)radiation, while markers involved in angiogenesis and hypoxia, COX-2, and serum SCC-ag levels are associated with a poor prognosis. Therefore targeting these pathways in combination with chemoradiation may improve survival in advanced stage cervical cancer patients.

Introduction

Cervical cancer is one of the most common malignancies among women worldwide (1). Standard treatment of locally advanced cervical cancer is concurrent platinum-based chemoradiation, resulting in a 5-year survival of only 66% (2). Currently, the most important prognostic factors in advanced stage cervical cancer primarily treated with (chemo)radiation are clinicopathological factors, including stage and tumor histology (3). Besides clinicopathological factors, many cell biological markers have been studied in relation to survival and/or response to (chemo)radiation. Especially markers involved in tumorigenesis and tumor progression, such as genes associated with apoptosis, angiogenesis and cell growth have been investigated extensively. At the moment the focus of improving survival rates is mainly on targeted therapies in combination with standard chemoradiation (4). Cell biological markers may be helpful to select patients who may benefit from additional treatment and in identifying new potential targets for therapy. Therefore, the aim of this systematic review was to identify prognostic and predictive cell biological markers in cervical cancer patients primarily treated with (chemo)radiation, and to review their potential application in treatment of advanced stage cervical cancer.

Methods

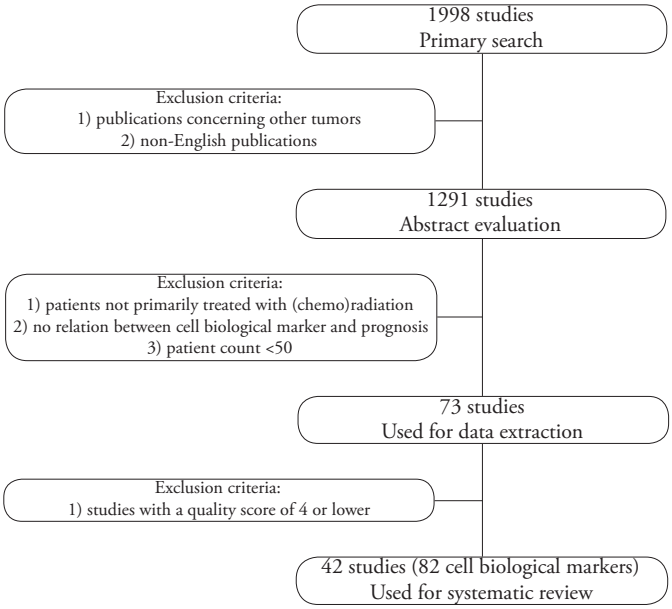
Search strategy

A PubMed, Embase and Cochrane literature search was performed on March 1st, 2010, to identify studies on prognostic cell biological markers in cervical cancer. Mesh-terms used for primary search were: Uterine Cervical Neoplasm, Biological Markers, Genes Neoplasm, Neoplasm Proteins, Prognosis and Tumor marker. References from included reviews were also hand-searched to identify missing relevant publications. The total number of identified publications was 1590 for PubMed, 808 for Embase and 0 for Cochrane. In PubMed and Embase 400 publications were identified in both databases, after extraction of these publications a total number of 1998 studies were identified (Fig. 1).

Selection criteria for data extraction

Based on article title, first abstracts were excluded, that concerned tumors other than cervical cancer (n=564). Secondly non-English publications were excluded (n=143). The total number of studies remaining for abstract evaluation was 1291. All abstracts were reviewed by two independent researchers (MGN and JJHE). Abstract selection

Figure 1 – Selection process



was based on the following criteria: 1) the study concerned cervical cancer patients primarily treated with (chemo)radiation, 2) a relation between a cell biological marker and survival was studied, and 3) a minimum patient count of n $\geq$50 (arbitrarily chosen). After that, 73 publications were selected for data extraction (Fig. 1).

Data extraction

Data were extracted by two independent researchers using a predefined form. Topics in this form were: year of publication, country, number of patients, years of patient inclusion, method of case selection (retrospective or prospective cohort of patients), age at time of diagnosis (mean, median, range), follow-up time (mean, median, range), distribution of stage, tumor type and differentiation grade, type of treatment, assay method and interpretation of assay used, number of marker positive and negative tumors, numbers of death, and results of univariate and/or multivariate survival analyses (log-rank analysis, Cox regression). A variety of endpoints were used to evaluate prognostic significance, including disease-specific survival (DSS), disease-free survival (DFS), overall survival (OS), and metastasis-free survival (MFS). As cell biological markers may be helpful to select patients who may benefit from additional treatment, markers associated with response to therapy may be even more interesting. Therefore, as a secondary

endpoint response to therapy was evaluated, defined as locoregional disease-free survival (LDFS), pathological response after treatment, or locoregional control.

Study quality assessment

The quality of studies was assessed by two independent researchers. Discordance between scores was reappointed on consensus of opinion. The quality measurement form was based on work from Hayes *et al.* and the REMARK (REporting recommendations for tumour MARKer prognostic studies) criteria from McShane *et al.* (5,6). In summary, the following criteria were investigated; whether 1) the study reported in- and exclusion criteria; 2) study data were prospectively collected; 3) 4 out of 6 of the following patients and tumor characteristics were described: a) age, b) stage, c) tumor histology, d) differentiation grade, e) lymphovascular space involvement, f) tumor size; 4.1) the assay used to measure biomarker expression was sufficiently described; 4.2) interpretation of the assay was described; 5) definition of the study endpoint was given; 6) the follow up time of patients in the study was described; 7) the study reported how many patients were available for statistical analysis. Studies with a total score of eight were considered to show the highest study quality, while a zero score indicated the lowest quality. Criteria 1 and 2 are strongly related to prospective studies, implying that most retrospective studies could only reach a maximum quality score of 6 (criteria 3 to 7). In order to be included in the systematic review it was allowed to miss only one of the remaining 6 criteria to ensure that these studies are providing real insight into the reliability of their data. Therefore, the cut-off value for studies to be included in the systematic review was set at a quality score of 5 or higher.

Data analysis

Only a small number of markers were investigated in more than one independent study, therefore cell biological markers were clustered on biological function. The following clusters were considered: angiogenesis and hypoxia markers, apoptosis markers, cyclooxygenase-2 (COX-2), Epidermal Growth Factor Receptor (EGFR)-pathway, human papilloma virus (HPV), proliferation markers, serum tumor markers, and miscellaneous cell biological markers. The prognostic significance of cell biological markers in each cluster will be described. A cell biological marker is considered as a potential marker for prognosis if >50% of the studies investigating the marker describe a relation with poor or good prognosis. After that, cell biological markers that were independently related to prognosis are described in more detail. If described in the study, the hazard

ratio and 95% confidence interval will be provided. Because of the small number of markers that were investigated in more than one independent study and the heterogeneity of these studies no meta-analysis was performed.

Results

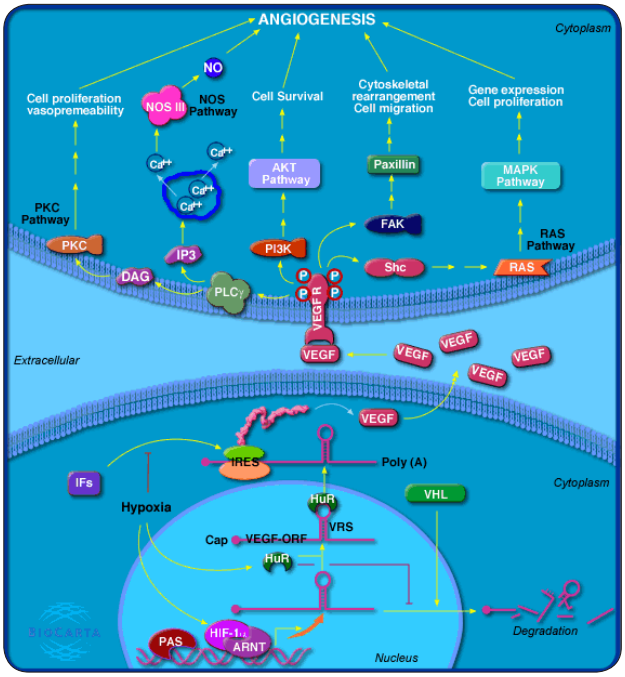
In total 42 studies concerning 82 cell biological markers were selected for this systematic review. In univariate analysis 34 cell biological markers showed a relation with survival and 27 were independently associated with survival. In the next subsections the prognostic significance of these cell biological markers will be described.

Angiogenesis and hypoxia markers

Angiogenesis is essential for growth and progression of cancer (Fig. 2) (7). Insufficient formation of new blood vessels leads to tumor hypoxia and limits cell growth. On the other hand, adaptation of the tumor to hypoxic conditions because of insufficient oxygenation may also lead to a more malignant phenotype or to poor response to treatment, such as radiotherapy (8,9). One of the most important regulators of response to hypoxic stress is hypoxia-inducible factor (HIF)-1α (10). In two out of three studies higher HIF-1α immunostaining was associated with poor survival (11-13). HIF-2α protein is closely related to HIF-1α, but was not related to survival (14). The HIF-2α/CD68 ratio was related to poor DFS, but not to DSS (14). Vascular endothelial growth factor (VEGF) can be upregulated in response to hypoxia, for example by HIF-1α and induces angiogenesis (7). Out of two studies on the prognostic significance of VEGF only one study found that VEGF immunostaining was related to poor OS and MFS (15,16). Thymidine phosphorylase (TP) levels can also increase under hypoxic conditions, and TP immunostaining was associated with poor DSS and MFS, but not to local control (17). Nitric oxide is required for angiogenesis and is produced by nitric oxide synthase (NOS), which in turn is induced by angiogenic factors, such as VEGF (18). Increased immunostaining of inducible NOS appears to be a poor prognostic factor (19). Finally, expression of carbonic anhydrases was investigated. Carbonic anhydrases can also be induced under hypoxic conditions by HIF-1α (20). Carbonic anhydrase 9 (CA9) immunostaining was investigated in three studies: Lee *et al.* found a relation with poor DFS, while the other studies showed a relation with poor DSS, MFS, and OS (15,21,22). Carbonic anhydrase 12 (CA12) expression was related to good MFS in multivariate but not in univariate analysis, in only one out of two studies (15,21). In conclusion, expression of multiple proteins involved in tumor angiogenesis or hy-

poxia has been studied and most proteins show a relation with poor survival. Especially expression of HIF-1α and CA9 are associated with poor prognosis.

Figure 2 – VEGF, hypoxia, and angiogenesis



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Apoptosis markers

Three studies in which frequency of apoptotic cells in pre-treatment cervical cancer tissue was determined by morphology, did not find a relation between numbers of apoptotic cells with survival (23-25). In addition, several studies evaluated the expression of proteins involved in apoptotic pathways in relation to survival. The Bcl-2 family is a key regulator in the apoptotic process; Bcl-2 is an inhibitor of apoptosis, while Bax and Bid are pro-apoptotic proteins (26). Bcl-2 was associated with poor DFS (only in multivariate analysis), while Bax was associated with good DFS (27). In contrast, Bid was associated with poor MFS (28). Apoptosis can also be induced by binding of TRAIL to its death receptors (DR), DR4, and DR5 (Fig. 3) (29). Immunostaining of TRAIL, DR4 and DR5 was not related to DSS (30). Furthermore, protein expression and mutational status of the pro-apoptotic *p53* have been studied. No relation between p53 immunostaining and survival was found, and in one out of two studies on

p53 mutations, a relation was found between presence of a mutation and poor survival (27,31-33). Finally, immunostaining of p63, a p53-related protein, was associated with poor survival (34). Overall, based on these studies there is no convincing evidence that apoptotic markers have prognostic significance in cervical cancer.

COX-2

COX-2 is an important enzyme in the conversion of arachidonic acid to prostaglandins, which is involved in the inflammatory process (35). Besides its role in inflammation, COX-2 is frequently overexpressed in (cervical) cancer and is associated with inhibition of apoptosis, and promotion of angiogenesis (36). Even more interesting especially in this patient population, is that COX-2 may play a role in response to radiotherapy, as COX-2 inhibition enhances tumor response to radiation, for example by inhibition of DNA damage repair after radiation *in vitro* (37). In all studies immunostaining of COX-2 was related to poor survival (19,38-40). Kim *et al.* described co-expression of EGFR and COX-2, which was related to poor survival (38). Overall, these studies all show that stronger COX-2 immunostaining has a negative prognostic impact and therefore COX-2 appears to be an important prognostic marker.

EGFR pathway

The EGFR pathway is often dysregulated in cancer (Fig. 4). It has been shown to be involved in processes associated with carcinogenesis and tumor progression, such as inhibition of apoptosis, cell migration, cell growth, and angiogenesis (41), and also in conferring resistance to irradiation (42,43). Protein expression of multiple genes involved in the EGFR pathway have been studied in relation to prognosis in cervical cancer. (phosphorylated)EGFR (HER1) and C-erbB-2 (HER2) immunostaining as well as co-expression of EGFR and C-erbB-2 are associated with poor DFS or DSS (3,34,38,44,45). There is less evidence for the prognostic significance of the other receptors of the EGFR family, as only Her3 and Her4 immunostaining were studied, of which Her4 was associated with good DFS, while no relation between Her3 and survival was found (46). AKT is a member of the PI3K/AKT pathway, which is activated by phosphorylation of EGFR (41). Lee *et al.* found phosphorylated AKT to be related with good DFS, but not with OS in 55 cervical cancer patients (46). In contrast, we found in a large series of cervical cancer patients (n=375) that pAKT does not have prognostic significance (3). Phosphorylation of EGFR also leads to activation of the ERK-pathway, but protein expression of phosphorylated ERK was also not related to

Figure 3 – Induction of apoptosis through DR3 and DR4/5 death receptors

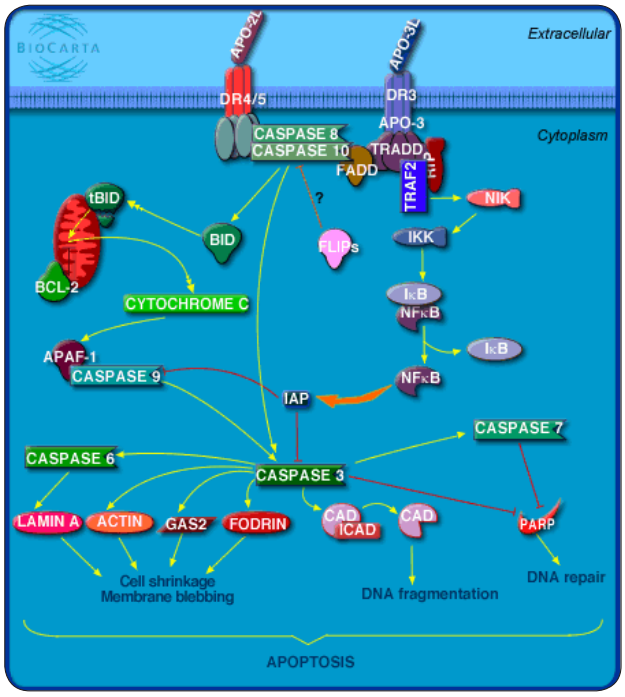

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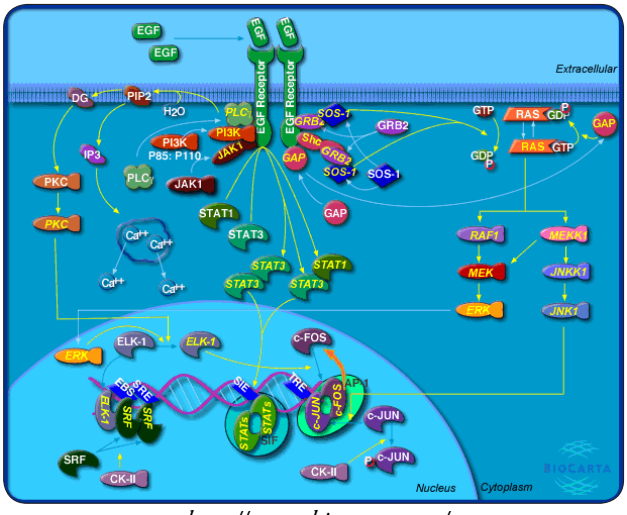
Figure 4 – EGF signaling pathway



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survival (3). Phosphatase and Tensin Homolog Deleted on Chromosome Ten (PTEN) acts as a tumor suppressor gene by inhibiting phosphorylation and thereby activation of AKT (47,48). Mutations in *PTEN* were associated with poor OS (49). However, immunostaining of PTEN was not associated with survival DSS and OS (3). In summary, there is convincing evidence that high protein expression of genes involved in the EGFR pathway, particularly EGFR and C-erbB-2, are indicators of poor survival in cervical cancer patients primarily treated with (chemo)radiation.

HPV

A causal role for HPV in the development of cervical cancer has been established (50). Since almost all cervical cancers are HPV positive, only HPV subtypes may be related to survival. Bachtiary *et al.* studied presence of HPV types 16, 18, 31, 33, 45, and other HPV types (HPV 35, 56, 58, and 76) (51) and reported only HPV 33 as a was a poor prognostic factor. However, presence of more than one of these HPV types (16, 18, 31, 33, 35, 45, 56, 58, and 76) was related to poor DSS and DFS survival (51). Ishikawa *et al.* also tested for presence of HPV types 16, 18, and 33 and found no relation with survival (33). Finally, Harima *et al.* found a relation between presence of HPV (type 6, 16, 18, 31, 33, 35, 52, 56, 58, or 59) and good OS and DFS (32). Because of the variety of HPV types studied and the discrepancies between studies the prognostic value of HPV remains doubtful.

Proliferation markers

Tumor proliferation can be determined by a variety of methods. The bromodeoxyuridine (BrdU) labelling method measures the labelling index (LI), which reflects proliferation. Tsang *et al.* found that BrdU LI as measured by flow cytometry was associated with poor DFS (not with OS), while BrDU LI as determined by histologic assessment did not have any prognostic impact (23). MIB-1 LI, which is an immunohistochemical marker of cell proliferation, was also not associated with OS (24). Surprisingly, a high mitotic index (MI) (which was determined based on morphologic criteria) was associated with good OS in one out of two studies (23,24). The ratio of the apoptotic index/mitotic index was associated with poor OS (24). No relation was found between duration of the S-phase and prognosis (23). Finally, a potential doubling time (Tpot) of tumor cells >5 days, as calculated from the BrdU LI and S-phase duration, was associated with good DFS (23). Overall, these studies do not provide convincing evidence for a prognostic role for markers associated with tumor proliferation.

Serum tumor markers

Serum tumor markers studied are squamous cell carcinoma antigen (SCC-ag) and carcinoembryonic antigen (CEA). CEA levels >10 ng/ml were associated with poor survival (52). Serum SCC-ag levels have been investigated in four studies by enzyme immunoassays and three out of four showed a relation between high serum SCC-ag levels and poor survival (52-55).

Miscellaneous cell biological markers

An overview of the prognostic value of other markers is given in Table 1. Only protein expression of the tumor suppressor gene Fragile Histidine Triad Protein (FHIT) was studied by two independent groups and one study showed that abnormal expression was a poor prognostic factor for OS (56,57).

Table 1 – Miscellaneous cell biological markers

First Author	Marker	Survival Univariate
Dickson (71)	TGF-β1	**OS** ↓
Ferrandina (58)	Class III β-tubulin	OS ↔, DFS ↔
Gruber (31)	β3-integrin	**DSS** ↓, **MFS** ↓
Harima (72)	LOH6p21.1	**OS** ↓, **DFS** ↓
Kawanaka (14)	CD68	DSS ↔, DFS ↔
Krivak (56)	FHIT	**OS** ↓
Kwon (59)	CD24	OS ↔, DFS ↔, **MFS**↓
Machida (57)	FHIT	OS ↔
Schindl (73)	KAI1	OS ↔, DFS ↔, MFS ↔
Suzuki (60)	Estrogen receptor	DFS ↔
Suzuki (60)	Progesterone receptor	**DFS** ↑

TGF-β1 = Transforming Growth Factor-β1; LOH6p21.1 = Loss of Heterozygosity of chromosome 6p21.1; CD68 = Cluster of Differentiation 68; FHIT = Fragile Histidine Triad Protein; CD24 = Cluster of Differentiation 24; KAI1 = also known as CD82; DSS = disease-specific survival; DFS = disease-free survival; OS = overall survival; MFS = metastasis-free survival; ↓ = positivity of marker associated with poor survival; ↑ = positivity of marker associated with good survival, ↔ = no relation with survival

Independent prognostic cell biological markers

In total, 36 cell biological markers were tested in a multivariate model. An independent association with survival was observed for 26/36 cell biological markers (Table 2). All cell biological markers were analyzed in multivariate models including classic prognostic factors. Therefore, these cell biological markers give additional information on prognosis in cervical cancer patients primarily treated with (chemo)radiation, complementary to clinicopathological prognostic factors.

Markers associated with response to (chemo)radiation

Associations with response to treatment as determined by LDFS, pathological response after treatment, or locoregional control, was evaluated for 38/82 markers and 14/38 markers were associated with poor response in univariate analysis. In multivariate analysis seven markers were also associated with poor response. An overview of these markers is given in Table 3. Markers involved in the EGFR pathway (EGFR and C-erbB-2) and COX-2 were associated with poor response to (chemo)radiation. Interestingly, most markers involved in angiogenesis and hypoxia markers (VEGF, TP, HIF-1α, CA9, and CA12), which did have prognostic significance, were not predictive for response to (chemo)radiation (12,13,15-17,22). Apoptosis markers (BID, p53, DR4, and DR5) (28,30,31), a few members of the EGFR pathway (C-erbB-2 in one study, pAKT, pERK, and PTEN) (3,45), HPV infection (33), and miscellaneous cell biological markers (Class III β-tubulin, CD24, FHIT, estrogen and progesterone reseptor) (57-60) did also not show a relation with response.

Discussion

This systematic review summarizes the prognostic and predictive value of cell biological markers in cervical cancer patients primarily treated with (chemo)radiation. Clusters with the strongest prognostic factors consist of markers involved in angiogenesis and hypoxia and markers involved in the EGFR pathway. Furthermore, COX-2 immunostaining and serum SCC-ag levels appear to be prognostic markers. Besides the prognostic significance, associations with response to (chemo)radiation were also studied for some markers to determine their predictive value. Interestingly, EGFR and C-erbB-2 were also associated with poor response to radiotherapy, while markers involved in angiogenesis and hypoxia did not show a relation with response.

In addition to the relevance of prognostic significance of individual cell biological markers, as shown above, it may be even more interesting that also relations exist between COX-2, the EGFR pathway, angiogenesis and hypoxia. One of the mechanisms in the relation between EGFR and COX-2, is that COX-2 derived prostaglandin E_2 (PGE_2) activates the EGFR pathway, which in turn results in increased COX-2 expression (61). In cervical cancer cell lines it was shown that EGF indeed can induce COX-2 protein expression (36). As pointed out in the results section, co-expression of EGFR and COX-2 was related to poor DFS in cervical cancer treated with chemoradiation (38). Furthermore, HIF-1α can induce expression of COX-2 under hypoxic conditions and the elevated levels of PGE_2 promote transcriptional activity of HIF-1α and expression of VEGF, also indicating a positive feedback mechanism (62).

As higher expression of the most interesting cell biological markers identified by this systematic review was associated with poor prognosis, they may be potential targets for therapeutic intervention. Targeted treatment for these markers is already under development. For example, EGFR targeted therapy in combination with standard radiotherapy has recently been implemented as a new therapeutic strategy in various malignancies (63). Clinical trials with the monocloncal anti-EGFR inhibitor, cetuximab in combination with (chemo)radiation in cervical cancer are ongoing (http://www.cancer.gov/clinicaltrials, NCT00104910, NCT00292955, NCT00957411). Celecoxib, a specific COX-2 inhibitor, has a radiosensitizing effect in various malignancies. However, celecoxib in combination with standard chemoradiation in cervical cancer caused major toxicity, while no beneficial effect on survival rates was observed (64,65). Because of the relations between the markers, as described above, also combinations of targeted therapies may be attractive. For instance, combining COX-2 and EGFR inhibitors showed synergistic effects in preclinical studies (66).

Not surprisingly, high pre-operative serum SCC-ag levels were found to be associated with poor prognosis. This is most probably because serum SCC-ag levels reflect classic prognostic factors, such as FIGO stage and tumor size (67). However, two studies reported prognostic significance independent of clinicopathological prognostic factors (54,55). Analysis of decline in serum SCC-ag levels during treatment has also been performed and appears to be indicative of tumor response to (chemo)radiation and outcome of patients (67).

Table 2 – Independent prognostic factors

First Author	Marker	Method	Cut-off	Patients	Treatment	Histology	Stage	Survival (HR; 95%CI)	
Angiogenesis and hypoxia markers									
Bachtiary (11)	HIF-1α	IHC	Moderate/strong	67	RT	SCC/AC	Ib-III	**DSS** ↓ (2.1; 1.0 – 4.1)	**DFS** ↓ (2.1; 1.1 – 4.2)
Burri (12)	HIF-1α	IHC	3 cat.: weak, moderate, strong	78	RT/CRT	SCC/AC	Ib-IVa	**OS** ↓ (1.6; 1.1 – 2.3)	
Loncaster (16)	VEGF	IHC	4 cat.: 0, 1, 2, 3	100	RT	SCC/AC	Ib-IIIb	**OS** ↓ (2.3)	**MFS** ↓ (2.1)
Chen (19)	iNOS	IHC	>15% cytoplasmic	167	RT/CRT	SCC	Ib-IV	**DFS** ↓ (2.0; 1.2 – 3.4)	
Lee (21)	CA9	IHC	Present	75	RT	SCC/AC	I-IV	**DFS** ↓ (6.1; 1.3 – 28.3)	
Loncaster (22)	CA9	IHC	Present	130	RT	SCC	I-IV	**DSS** ↓	
Kim (15)	CA9	PCR	Present	59	RT	SCC	I-IV	**MFS** ↓ (34.8; na)	OS ↔
Kim (15)	CA12	PCR	Present	59	RT	SCC	I-IV	**MFS** ↑ (0.1)	OS ↔
Apoptosis markers									
Wootipoom (27)	Bax	IHC	>50% 1+ / <50% 2+	174	RT	SCC	Ib-IVa	**DFS** ↑ (0.5; 0.3 – 0.9)	OS ↔
Wootipoom (27)	BCL-2	IHC	>50%	174	RT	SCC	Ib-IVa	**DFS** ↓ (2.5; 1.0 – 6.1)	OS ↔
Cho (34)	p63	IHC	>15% nuclear	84	CRT	SCC	IIb	**OS** ↓ (3.2; 2.0 – 4.4)	
COX-2									
Kim (39)	COX-2	IHC	>50%	105	CRT	SCC/AC	IIb	**OS** ↓ (2.6; 1.8 – 3.4)	**DFS** ↓ (2.9; 2.1 – 3.7)
Chen (19)	COX-2	IHC	% x intensity >100	167	RT/CRT	SCC	Ib-IV	**DFS** ↓ (2.0; 1.2 – 3.4)	
Kim (38)	EGFR/COX-2 coexpr.	IHC	>10%	68	CRT	SCC	IIb	**DFS** ↓ (4.0; 2.7 – 5.3)	
EGFR pathway									
Perez-Regadera (44)	EGFR/C-erbB-2 coexpr.	IHC	EGFR >30%+, C-erbB-2 >69%+	170	RT/CRT	SCC/AC	Ib-IVa	**DFS** ↓ (2.9;1.3 – 6.7)	
Noordhuis (3)	EGFR	IHC	>10% membranous	375	RT/CRT	SCC/AC	Ib-IVa	**DSS** ↓ (1.5; 1.1 – 2.2)	

Table 2 – (continued)

First Author	Marker	Method	Cut-off	Patients	Treatment	Histology	Stage	Survival (HR; 95%CI)	
HPV									
Bachtiary (51)	≥2 HPV types	PCR	Present	96	RT	SCC/AC	Ib-IIIb	**DSS** ↓ (2.4; 1.3 – 4.5)	**DFS** ↓ (1.9; 1.0 – 3.5)
Harima (32)	HPV	PCR	Present	84	RT	SCC/AC	Ib-IVb	**OS** ↑ (0.43; na)	**DFS** ↑ (0.36)
Proliferation markers									
Gasinska (24)	Mitotic index	IHC	>2.6	130	RT	Unknown	I-III	**OS** ↑	
Gasinska (24)	AI/MI ratio	IHC	>0.7	130	RT	Unknown	I-III	**OS** ↓	
Serum tumor markers									
Chen (52)	CEA	EIA	>10 ng/ml	148	CRT	SCC	Ib-IVa	**DSS** ↓ (3.2; 1.2 – 9.4)	**DFS** ↓ (2.6; 1.1 – 7.9)
Hong (55)	SCC-ag	EIA	3 cat: <2, 2-10, >10 ng/ml	401	RT	SCC	I-IVa	**DSS** ↓ (2.5)	
Ogino (54)	SCC-ag	EIA	>6 ng/ml	359	RT	SCC	IIb-IV	**OS** ↓ (1.7; 1.2 – 2.4)	**DFS** ↓ (1.8; 1.2 – 2.7)
Miscellaneous cell biological markers									
Gruber (31)	β3-integrin	IHC	Weak and strong	82	RT	SCC/AC	I-IV	**DSS**↓ (4.4; 1.7 – 11.0)	**MFS** ↓ (4.0; 1.3 – 12.4)
Krivak (56)	FHIT	IHC	Abnormal	59	RT	SCC	II-III	**OS** ↓ (2.8; 1.2 – 7.8)	
Kwon (59)	CD24	IHC	>50%	73	RT	SCC	I-IV	**MFS** ↓	OS, DFS ↔

HIF-1α = Hypoxia-inducible factor-1α; VEGF = Vascular endothelial growth factor; iNOS = Inducible nitric oxide synthase; CA9 = Carbonic anhydrase 9; CA12 = Carbonic anhydrase 12; Bax = BCL-2-associated X protein; BCL-2 = B-cell lymphoma 2; p63 = tumor protein 63; COX-2 = Cyclooxygenase-2; EGFR = Epidermal Growth Factor Receptor, also known as HER1; c-erbB-2 = also known as HER2; HPV = Human papillomavirus; AI/MI = apoptotic index/mitotic index; CEA = Carcinoembryonic Antigen; SCC-ag = Squamous Cell Carcinoma antigen; IHC = immunohistochemistry; PCR = Polymerase chain reaction; EIA = Enzyme immunoassay; cat. = categories; RT = radiotherapy; CRT = chemoradiation; SCC = squamous cell carcinoma; AC = adenocarcinoma; HR = Hazard ratio; 95%CI = 95% confidence interval; na = not available. Other abbreviations as in Table 1. Arrows as defined in Table 1.

Table 3 – Markers associated with response to treatment

First Author	Marker	Method	Cut-off	Patients	Treatment	Histology	Stage	Response (OR/HR; 95%CI)	
								Univariate	Multivariate
Angiogenesis and hypoxia markers									
Bachtiary (11)	HIF-1α	IHC	Moderate/strong	67	RT	SCC/AC	Ib-III	**Response** ↓	
Kawanaka (14)	HIF-2α/CD68 ratio	IHC	$\geq$22	73	RT/CRT	SCC/AC	Ib-IVb	**Response** ↓	
Apoptosis markers									
Cho (34)	p63	IHC	>15% nuclear	84	CRT	SCC	IIb	**Response** ↓	
Ishikawa (33)	p53 mutations	PCR	Present	52	RT	SCC	IIIb	**LDFS** ↓	
Maduro (30)	TRAIL	IHC	>weak positive	332	RT/CRT	SCC/AC	Ib-IVa	Response ↔	**Response** ↓ (2.1; 1.0 – 4.3)
COX-2									
Kim (39)	COX-2	IHC	>50%	105	CRT	SCC/AC	IIb	**Response** ↓	**Response** ↓
Kim (38)	EGFR/COX-2 coexpr.	IHC	>10%	68	CRT	SCC	IIb	**Response** ↓	
EGFR pathway									
Perez-Regadera (44)	C-erbB-2	IHC	>69%+	170	RT/CRT	SCC/AC	Ib	**LDFS** ↓	
Perez-Regadera (44)	EGFR	IHC	>30%+	170	RT/CRT	SCC/AC	Ib	**LDFS** ↓	
Perez-Regadera (44)	EGFR/C-erbB-2 coexpr.	IHC	EGFR >30%+, C-erbB-2 >69%+	170	RT/CRT	SCC/AC	Ib	**LDFS** ↓	**LDFS** ↓ (4.0; 1.4 – 11.1)
Noordhuis (3)	EGFR	IHC	>10% membranous	375	RT/CRT	SCC/AC	Ib-IVa	**LDFS** ↓ (1.9; 1.2 – 2.6)	**LDFS** ↓ (1.8; 1.2 – 2.8)
Noordhuis (3)	pEGFR	IHC	>1+ cytoplasmic	375	RT/CRT	SCC/AC	Ib-IVa	**LDFS** ↓ (2.1; 1.4 – 3.2)	**LDFS** ↓ (1.7; 1.1 – 2.7)

Table 3 – (continued)

First Author	Marker	Method	Cut-off	Patients	Treatment	Histology	Stage	Response (OR/HR; 95%CI)	
								Univariate	Multivariate
Proliferation markers									
Bolger (74)	Adjusted brdUrd LI	FC	<median	121	RT	SCC/AC	I-IV	**Response** ↓ (1.1; 1.0 – 1.2), **LDFS** ↓	**Response** ↓ (1.1; 1.0 – 1.2)
Miscellaneous cell biological markers									
Gruber (31)	β3-integrin	IHC	weak and strong	82	RT	SCC/AC	I-IV	**LDFS** ↓	**LDFS** ↓ (6.8; 2.2 – 21.0)
Dickson (71)	TGF-β1	Elisa	4.26 ng/ml	79	RT	SCC/AC	I-III	**LDFS** ↓	

HIF-2α = Hypoxia-inducible factor-2α; p53 = tumor protein 53; TRAIL = Tumor necrosis factor Related Apoptosis Inducing Ligand; pEGFR = phosphorylated Epidermal Growth Factor Receptor; Adjusted brdUrd LI = Adjusted bromodeoxyuridine labelling index; FC = flow cytometry; OR = Odds ratio; LDFS = locoregional disease-free survival; ↓ = positivity of marker associated with poor response; ↔ = no relation with response. Other abbreviations as in Table 1 and 2.

From the 1998 papers that were found by the initial literature search, only 42 were included in this systematic review. This extreme fall in number of studies is mainly caused by heterogeneity of primary treatment modality in the study population; only a small number of studies was performed in patients primarily treated with (chemo)radiation or allowed evaluation of this population separately. We choose not to include studies both on surgically and (chemo)radiotherapeutically treated patients, as surgically treated patients do have other clinicopathological prognostic factors (e.g. presence of lymph node metastases, depth of tumor invasion) that play a role in clinical decision making. More importantly, other cell biological markers may be important in these groups, as for surgically treated patients predictors of lymph node metastases are more important, while in patients treated with (chemo)radiation e.g. the effect of the cell biological marker on response to (chemo)radiation is more relevant.

Despite the fact that this systematic review only includes well-documented studies on prognostic markers in a relatively homogeneous patient population, it was still difficult to compare studies analyzing the same cell biological marker. First, many studies did not perform a multivariate analysis or did not report hazard ratios and confidence intervals, thereby not allowing a meta-analysis for cell biological markers that have been described more than once. Second, different conclusions can be drawn from studies reporting on the same cell biological marker, mainly because of differences in methodology used in these studies (5). For example different antibodies are used for immunohistochemical staining and, more important, most studies do not use similar cut-offs for positivity of the marker and even do not give a rationale for a certain cut-off score.

The lack of reporting information important for interpretation of the study is a well known problem in studies on prognostic tumor markers (68). Although we only selected studies with a certain quality for this systematic review, many studies did not fulfill all of the criteria from the quality measurement. Especially, many studies did not report in- and exclusion criteria and collected patient material retrospectively. In future, quality of studies on prognostic markers could be improved by performing well-documented prospective studies including a large number of patients, which should completely fulfill the REMARK criteria (5). Improving quality of studies reporting prognostic markers, should lead to more reliable conclusions about the prognostic significance of cell biological markers.

The majority of studies included in this systematic review focused on one or two related cell biological markers. However, markers can also be investigated by microarray-technology, which allows measurement of global gene expression in only one experiment. Various microarray studies have already been performed in advanced stage cer-

vical cancer patients. For example, genes related to local failure and distant metastasis in cervical cancer patients treated by radiotherapy were identified, but these genes did not overlap with the markers identified by this systematic review (69). Harima *et al.* identified genes related to local failure after thermoradiotherapy in cervical cancer patients, and found hypoxia-inducible genes (HIF-1α and CA12) to be higher expressed in patients who had local recurrences (70), again confirming the important role of this pathway in response to radiotherapy.

In conclusion, our systematic review identified prognostic and predictive cell biological markers in cervical cancer patients primarily treated with (chemo)radiation. The most interesting markers that were identified were markers involved in EGFR signaling, angiogenesis, hypoxia and COX-2. Besides their prognostic significance, EGFR, C-erbB-2 and COX-2 were also associated with poor response to (chemo)radiation. Further research of these markers and their targeting by targeted drugs in combination with chemoradiation in clinical practice will hopefully improve survival rates in advanced stage cervical cancer patients.

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The epidermal growth factor receptor pathway in relation to pelvic lymph node metastasis and survival in early stage cervical cancer

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Abstract

Objective: To correlate the expression of EGFR components with clinical behavior of early stage cervical cancer.

Patients and methods: Tissue samples of 336 consecutive FIGO stage Ib-IIa cervical cancer patients all treated primarily by radical surgery were collected. Clinicopathological and follow-up data were prospectively obtained during standard treatment and follow-up. As representants for the EGFR pathway, expression of EGFR, pEGFR, PTEN, pAKT, and pERK was assessed by immunohistochemistry on tissue microarrays (TMAs).

Results: Positive immunostaining was observed for EGFR in 32.1%, for pEGFR in 21.0%, for PTEN in 38.3%, for pAKT in 5.3% and for pERK in 4.3% of tumor samples. Positive EGFR immunostaining was associated with squamous cell carcinoma of the cervix (OR=7.41; 95%CI=3.38 – 16.23; P<0.001), negative pEGFR immunostaining with poor differentiation (OR=0.39; 95%CI=0.20 – 0.73; P=0.004) and negative PTEN immunostaining with metastatic pelvic lymph nodes (OR=0.51; 95%CI=0.30 – 0.90; P=0.019). In multivariate analysis only pelvic lymph node metastasis (HR=6.11; 95%CI=3.46 – 10.77; P<0.001) and poor differentiation (HR=1.91; 95%CI=1.12 – 3.26; P=0.018) were related to disease-specific survival.

Conclusion: In early stage cervical cancer loss of PTEN expression is associated with pelvic lymph node metastasis, suggesting PTEN to be one of the tumor suppressor genes affecting pelvic lymph node metastasis. However, expression of EGFR pathway components does not appear to have prognostic impact in surgically treated early stage cervical cancer.

Introduction

Early stage cervical cancer is generally treated by radical hysterectomy and pelvic lymph node dissection. In cases with poor clinico-pathological factors, adjuvant radiotherapy with or without chemotherapy is often administered. Conventional prognostic factors in early stage cervical cancer are: tumor size, depth of stromal invasion, lymphovascular space involvement, parametrial invasion and pelvic lymph node metastasis (1-4). Pelvic lymph node metastasis appears to be the most important of these parameters (5), with a five-year survival approximating 90% in node negative early stage cervical cancer patients primarily treated with surgery and decreasing to approximately 65% in patients with pelvic lymph node metastasis (6). In early stage cervical cancer, molecular markers could be helpful in selecting lymph node negative patients with an unfavourable prognosis for adjuvant treatment and might identify new targets for patient-tailored therapy.

The epidermal growth factor receptor (EGFR) is involved in the ErbB signaling network, which is often deregulated in cancer. Autophosphorylation of EGFR to pEGFR leads to activation of the Ras/Raf/MEK/ERK pathway and the PI3K/AKT pathway, both of which are involved in processes that are associated with carcinogenesis and tumor progression, such as inhibition of apoptosis, cell migration, cell growth, and angiogenesis (7). PTEN (phosphatase and tensin homolog deleted on chromosome ten) acts as a tumor suppressor gene by inhibiting phosphorylation and thereby activation of AKT (8,9). Only few data, obtained in small series of cervical cancer patients primarily treated by surgery, exist on EGFR or PTEN expression (10-13) and no published studies provide a comprehensive analysis of several EGFR pathway components simultaneously in a well-defined series of early stage cervical cancer patients.

Previous studies of molecular markers in early stage cervical cancer have been limited by the small number of patients evaluated and/or mediocre documentation of clinico-pathological parameters. The present study was designed to correlate expression of EGFR, pEGFR, PTEN, pAKT, and pERK in relation to clinico-pathological parameters and disease-specific survival in a large, well-documented series of patients with early stage cervical cancer and long-term follow-up.

Patients and methods

Patients and treatment

Since 1980, clinico-pathological characteristics and follow-up data of all cervical cancer patients referred to the Department of Gynaecologic Oncology of the University Medical Center Groningen have been prospectively entered into a computerized database. Clinical staging of each patient is performed under general anaesthesia in accordance with the International Federation of Gynaecology and Obstetrics (FIGO) criteria. For the present study we selected all early stage cervical cancer patients, treated primarily by surgery between January 1980 and December 2004 from our database (n=336). All patients underwent type 3 radical hysterectomy and pelvic lymph node dissection. Patients with pelvic lymph node metastases, parametrial invasion or positive excision margins received adjuvant external beam radiotherapy with or without chemotherapy. Paraffin-embedded formalin-fixed primary tumor tissue was collected from each patient. Patients were only included in our analysis, if sufficient, representative tumor tissue was available for TMA construction. After completion of treatment, patients were followed up at the outpatient clinic for at least 5 years.

Institutional Review Board approval

In the University Medial Center Groningen clinico-pathologic and follow-up data are prospectively obtained during standard treatment and follow-up and stored in a computerized registration database. For the present study, all relevant data were retrieved from this computerized database into a separate, anonymous database. Patient identity was protected by study-specific, unique patient numbers. Codes were only known to two dedicated data managers, who also have daily responsibility for the larger database. In case of uncertainties with respect to clinico-pathologic and follow-up data, the larger databases could only be checked through the data managers, thereby ascertaining the protection of patients' identity. Using the registration database all tissue specimens were identified by unique patient numbers and retrieved from the archives of the Department of Pathology. Therefore, according to Dutch law no further Institutional Review Board approval was needed for this study (http://www.federa.org/).

Tissue Microarray (TMA) construction

As previously described, representative areas of tumor were marked on hematoxylin- and eosin- (H&E) stained slides of the paraffin-embedded tissue (14). Areas of necrosis

and/or heavy leucocytic infiltrate were avoided. The TMAs were constructed using a precision instrument (Beecher Instruments, Silver Spring, Maryland). Three 0.6 mm in diameter cores were punched from the marked area of the paraffin-embedded tissue (donor block) and transferred to a predefined location in a blank paraffin block (recipient block). After all the cores had been inserted, the recipient block was placed in an oven of 37ºC for 15 minutes to attach the cores to the surrounding paraffin. Each TMA also contained benign (skin epithelia, normal cervical tissue and colon polyps) and tumor (breast, colon and ovarian carcinoma) tissue that served as controls for immunostaining and comparison of TMAs. In total 5 TMAs were constructed.

Immunohistochemistry

For immunohistochemistry, 4 μm sections were cut from the TMA and mounted on amino-propyl-ethoxy-silan (APES, sigma-Aldrich, Diesenhofen Germany)-coated glass slides. Immunohistochemistry for EGFR, pEGFR, PTEN, pAKT, and pERK was performed as described previously (15). Details of the antibodies used for immunohistochemistry and methods for antigen retrieval are summarized in Table 1. The avidin-biotin-peroxidase method was utilized for all antibody detection, except pAKT for which the EnVision horseradish peroxidase system (Dako, Copenhagen, Denmark) was used. Slides were deparaffinized in xylene and rehydrated in ethanol. Endogenous peroxidase was blocked by incubation with 0.3% hydrogen peroxidase for 30 minutes. For stainings in which the avidin-biotin-peroxidase method was used, endogenous avidin and biotin activity was blocked using a blocking kit (Vector Laboratories, Burlingame, UK). Immunostaining was visualized by 3'3-diaminobenzidinetetrahydrochloride and counter immunostaining was performed with hematoxylin.

Evaluation of immunostaining

Scoring was performed by two independent observers (JJHE, MGN) without knowledge of clinical data. A concordance of more than 90% was found. The discordant cases were reviewed and scores were reassigned on consensus of opinion. Immunostaining intensity was semi quantitatively scored. Only patients with at least two representative cores were included in the analysis. Tumors were considered positive for EGFR in when >10% positive membranous immunostaining was observed (12). pAKT and pERK immunostaining were considered positive if >10% of a tumor showed cytoplasmic and/or nuclear immunostaining (15). Positive expression of PTEN was defined as >10% cytoplasmic immunostaining (16). Positive pEGFR was defined as at least weak positive cytoplasmic immunostaining, as the activated EGFR is internalized (17).

Table 1 – Antibodies utilized for immunostaining

Antigen	Antigen retrieval	Clone	Company	Dilution	Incubation time
PTEN	Citrate (pH 6)*	6H2.1	Cascade†	1:100	60 minutes
EGFR	Proteinase K 0.1%, 30 minutes	111.6	Neomarkers‡	1:200	60 minutes
pEGFR	EDTA (pH 8)*	1H12	Cell Signaling§	1:200	60 minutes
pAKT 1/2	Citrate (pH 6)*	736E11	Cell Signaling§	1:50	overnight (4ºC)
pERK 1/2	Citrate (pH 6)*	20G11	Cell Signaling§	1:50	overnight (4ºC)

*Sections were boiled in a microwave for 15 minutes
†Cascade Bioscience, Winchester, USA
‡Neomarkers, Lab Vision Corporation, Fremont, USA
§Cell signaling, Danvers, USA

Table 2 – Patient and tumor characteristics

	n=310	
	n	*%*
FIGO stage		
Ib1	197	64%
Ib2	63	20%
IIa	50	16%
Treatment		
WM*	188	61%
WM* + radiotherapy	106	34%
WM* + chemoradiation	16	5%
Histology		
Squamous carcinoma	200	65%
Adenocarcinoma	87	28%
Other	23	7%
Differentiation grade		
Good/moderate	180	58%
Poor	124	40%
unknown	*6*	*2%*
Lymphangioinvasion		
Yes	160	52%
No	149	48%
unknown	*1*	*0%*

	n=310	
	n	*%*
Depth of invasion		
0-10 mm	165	53%
≥10 mm	131	42%
unknown	*14*	*5%*
Margins		
Negative	299	96%
Positive	11	4%
Lymph nodes		
Negative	222	72%
Positive	88	28%
Tumor diameter		
0-4 cm	223	72%
≥4 cm	87	28%
Recurrence		
No	245	79%
Locoregional	38	13%
Distance	14	5%
Progression	4	1%
unknown	*9*	*5%*

*Wertheim Meigs

Statistical analysis

Statistical analysis was performed with SPSS 16.0 for Windows (SPSS Inc., Chicago, IL). Associations (Odds Ratios and 95%CI) between immunostaining intensity and clinico-pathological characteristics were assessed in a univariate logistic regression model using positive protein expression as dependent factor and the clinico-pathological characteristics as independent factors. Relations (Hazard Ratios and 95%CI) between disease-specific survival, clinico-pathological features and immunostaining were calculated using both univariate and multivariate Cox proportional hazard analysis. In these analyses, factors with a *P* value >0.10 in the univariate analyses were excluded stepwise in multivariate analyses. *P* values of <0.05 were considered statistically significant.

Results

Patients and tumor characteristics

In total 336 patients diagnosed with early stage cervical cancer (Ib1: n=221 (66%); Ib2: n=63 (19%); IIa: n=52 (15%)) and treated by radical hysterectomy and pelvic lymph node dissection were identified. In 310 cases sufficient pre-treatment tissue was available for TMA construction. Median age was 43 years (range 17-86 years) and median follow-up time was 5.5 years (range 0.31 – 18.60 years). Overall 5-year disease-specific survival for the 310 patients was 82.4%. Additional patient and tumor characteristics are summarized in Table 2.

Clinico-pathological factors in relation to EGFR, pEGFR, PTEN, pAKT, and pERK

The proportion of patients included in the analysis based on two or more representative cores was 93.5% for EGFR, 93.5% for pEGFR, 91.0% for PTEN, 90.7% for pAKT and 89.1% for pERK. Fig. 1 shows a representative negative and positive core for each staining. Positive EGFR immunostaining was observed in 93/290 (32.1%) patients, positive pEGFR immunostaining in 61/290 (21.0%) patients, positive PTEN immunostaining in 108/282 (38.3%) patients, positive pAKT immunostaining in 15/281 (5.3%) patients and positive pERK immunostaining in 12/276 (4.3%) patients. Positive pEGFR immunostaining correlated with PTEN (OR=2.45; 95%CI=1.37 – 4.40; *P*=0.003) and with pERK (OR=4.47; 95%CI=1.38 – 14.47; *P*=0.013). No additional correlations between the immunostains were found (data not shown).

Figure 1 – Tumor microarray stained for EGFR, pEGFR, PTEN, pAKT, and pERK at 100x magnification.

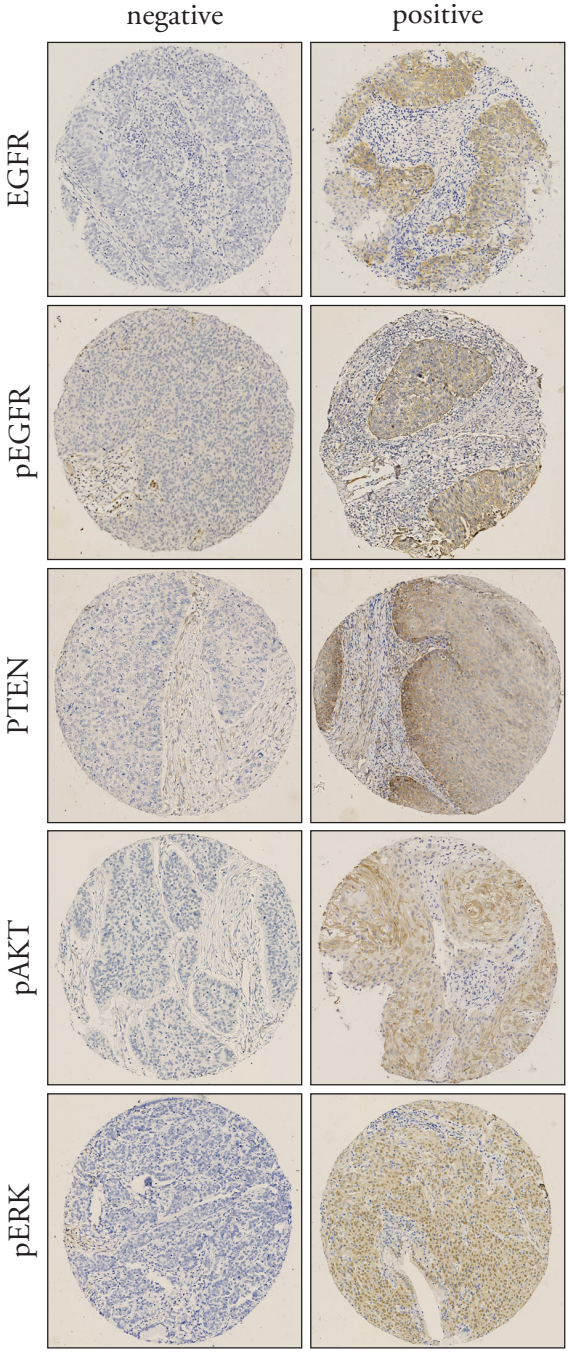

Table 3 shows clinico-pathological features in relation to immunostaining. Positive membranous immunostaining for EGFR was associated with squamous cell carcinoma of the cervix (OR=7.41; 95%CI=3.38 – 16.23; *P*<0.001), negative pEGFR cytoplasmic immunostaining was associated with poor differentiation (OR=0.39; 95%CI=0.20 – 0.73; *P*=0.004) and negative PTEN immunostaining was associated with metastatic pelvic lymph nodes (OR=0.51; 95%CI=0.30 – 0.90; *P*=0.019).

EGFR, pEGFR, PTEN, pAKT, and pERK and disease-specific survival

In univariate Cox Regression analysis, none of the immunostain results correlated with disease-specific survival (Table 4). In multivariate Cox Regression analysis only positive lymph nodes (HR=6.11; 95%CI=3.46 – 10.77; *P*<0.001) and poor differentiation (HR=1.91; 95%CI=1.12 – 3.26; *P*=0.018) were independent prognostic factors for disease-specific survival.

Discussion

Immunohistochemical expression of EGFR pathway components EGFR, pEGFR, PTEN, pAKT, and pERK was evaluated in relation to clinico-pathological parameters and disease-specific survival in a consecutive series of early stage cervical cancer patients. Our study indicates that loss of PTEN expression frequently occurs in early stage cervical cancer and is related to pelvic lymph node metastasis (OR=0.51; 95%CI=0.30 – 0.90; *P*=0.019), but not to survival. Only limited data exist on PTEN expression and its possible implications for the biologic behavior of cervical cancer. In a study by Lee *et al.* reduced PTEN expression was identified in 17.6% (15/85) of surgically treated cervical cancer patients and was associated with decreased disease-free and overall survival, but not with pelvic lymph node metastasis (13). They reported a gradual reduction of PTEN expression along the continuum from normal epithelium through intraepithelial neoplasia to squamous cell carcinoma. Discrepancies between the data from Lee *et al.* and our study might be due to the number of evaluated patients (n=85 vs. n=310) and/or interpretation of immunostaining, since Lee *et al.* defined reduced PTEN expression by comparison with corresponding normal tissue (13). In our opinion, a minimal percentage of positive cells should be taken into account when assessing PTEN expression in a tumor. In a study comparing different immunostainings in TMA and full sections of vulvar cancer patients, a minimal percentage of positive cells was also

Table 3 – Clinico-pathological parameters and immunostaining

	*OR**	*(95%CI) †*	*P value*	*OR**	*(95%CI) †*	*P value*
	EGFR positive			**pEGFR positive**		
Age	1.00	(0.99 – 1.02)	0.747	1.02	(0.99 – 1.04)	0.117
Stage ≥Ib2	1.37	(0.83 – 2.27)	0.222	0.87	(0.48 – 1.58)	0.653
Squamous	**7.41**	**(3.38 – 16.23)**	**<0.001**	1.41	(0.73 – 2.75)	0.325
Poor differentiation	0.75	(0.45 – 1.25)	0.273	**0.39**	**(0.20 – 0.73)**	**0.004**
Lymphangioinvasion	1.18	(0.72 – 1.94)	0.517	0.99	(0.56 – 1.75)	0.967
Infiltration depth ≥10 mm	1.50	(0.90 – 2.49)	0.120	0.78	(0.43 – 1.40)	0.407
Positive lymph nodes	1.62	(0.95 – 2.75)	0.077	1.20	(0.66 – 2.21)	0.547
Tumor diameter ≥4 cm	1.36	(0.80 – 2.34)	0.260	0.81	(0.42 – 1.54)	0.513
	PTEN positive			**pAKT positive**		
Age	1.00	(0.98 – 1.01)	0.630	1.02	(0.99 – 1.06)	0.248
Stage ≥Ib2	0.73	(0.44 – 1.21)	0.221	2.02	(0.71 – 5.75)	0.186
Squamous	1.76	(0.99 – 3.10)	0.052	2.33	(0.50 – 10.75)	0.280
Poor differentiation	0.66	(0.40 – 1.09)	0.102	0.74	(0.25 – 2.24)	0.597
Lymphangioinvasion	0.83	(0.51 – 1.34)	0.445	1.02	(0.36 – 2.90)	0.970
Infiltration depth ≥10 mm	0.61	(0.37 – 1.01)	0.053	0.38	(0.12 – 1.23)	0.106
Positive lymph nodes	**0.51**	**(0.30 – 0.90)**	**0.019**	0.58	(0.16 – 2.12)	0.410
Tumor diameter ≥4 cm	0.73	(0.42 – 1.25)	0.247	1.30	(0.43 – 3.92)	0.645
	pERK positive					
Age	0.99	(0.94 – 1.03)	0.550			
Stage ≥Ib2	0.14	(0.02 – 1.10)	0.062			
Squamous	0.61	(0.17 – 2.22)	0.452			
Poor differentiation	0.73	(0.22 – 2.50)	0.620			
Lymphangioinvasion	0.66	(0.20 – 2.12)	0.483			
Infiltration depth ≥10 mm	0.85	(0.26 – 2.73)	0.778			
Positive lymph nodes	0.83	(0.22 – 3.13)	0.777			
Tumor diameter ≥4 cm	0.00	(0.00 – 0.00)	0.997			

*Odds ratio
†95% confidence interval

taken into account and this study resulted in a good reproducibility of immunostaining on TMA (18).

Experimental data also indicate a role for loss of PTEN in determining the metastatic potential of tumors. In a study utilizing a benign melanocytic hyperplasia mice model, silencing PTEN lead to the development of melanoma and metastases to lymph nodes and lungs (19). In a study of colorectal cancer patients, Sawai *et al.* observed an association between reduced PTEN expression and liver metastases (20). Activation of receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor, Her2/neu and insulin-like growth factor receptor 1 results in recruitment of phosphoinositide 3-kinase

Table 4 – Disease-specific survival (Cox regression analysis)

	Univariate			Multivariate		
	*HR**	*(95%CI)* †	*P value*	*HR**	*(95%CI)* †	*P value*
Age	1.02	(1.00 – 1.04)	0.055	‡		
Stage ≥Ib2	**2.34**	**(1.38 – 3.98)**	**0.002**	‡		
Squamous	0.72	(0.40 – 1.29)	0.266	‡		
Poor differentiation	**1.73**	**(1.01 – 2.95)**	**0.045**	**1.91**	**(1.12 – 3.26)**	**0.018**
Lymphangioinvasion	**2.38**	**(1.32 – 4.26)**	**0.004**	‡		
Infiltration depth ≥10 mm	**2.00**	**(1.15 – 3.49)**	**0.014**	‡		
Positive lymph nodes	**5.76**	**(3.30 – 10.04)**	**<0.001**	**6.11**	**(3.46 – 10.77)**	**<0.001**
Tumor diameter ≥4 cm	**2.45**	**(1.44 – 4.16)**	**0.001**	‡		
EGFR positive	1.24	(0.69 – 2.22)	0.468	‡		
pEGFR positive	0.81	(0.39 – 1.66)	0.556	‡		
PTEN positive	0.61	(0.33 – 1.14)	0.120	‡		
pAKT positive	0.71	(0.17 – 2.93)	0.638	‡		
pERK positive	0.05	(0.00 – 15.02)	0.298	‡		

*Hazard ratio
†95% confidence interval
‡Not included in final analysis

(PI3K) (9). The direct product of PI3K is phosphatidylinositol-3,4,5-triphosphate (PIP3) and PIP3 is the primary target of PTEN (21). Loss of PTEN function results in accumulation of PIP3 and delete thereby activation of its downstream targets: the AKT pathway. Activation of the AKT pathway may cause cell cycle progression, cell survival, cell spreading and motility and angiogenesis (9). In our study, no relation between loss of PTEN and activation of the AKT pathway was found. Our low percentage (5.3%) of pAKT positive cases is in contrast to previous studies in cervical cancer that used the same antibody and staining protocol but observed 29-94% pAKT positive cases (22-24). A major difference between these studies and our study is that we assessed pAKT in a much larges series (310 vs. 31) of early stage cervical cancer patients.

Loss of PTEN can be due to mutations, deletions, gene promoter methylation or microRNAs (miRs) (25-28). Mutations and deletions of *PTEN* are rare events in cervical cancer (16,22,29). In a study by Yang *et al. PTEN* methylation was observed in 20/127 (15.7%) cervical cancers, while Cheung *et al.* reported *PTEN* methylation in 36/62 (58%) of squamous cell cervical cancers. They found no PTEN expression in 3 of 10 *PTEN* methylation negative cases and in 0 of 10 *PTEN* methylation positive cases (16). Preliminary data from our cases show *PTEN* gene promoter methylation in 4/19 (21%) cases. No PTEN immunostaining was observed in these 4 *PTEN* methylation positive cases (data not shown). Loss of PTEN expression could also occur via

miRs. Several miRs, such as miR-21 and miR-214, can target PTEN (27,28). Both miRs appear to be up-regulated in cervical cancer (30,31), but a relation with PTEN loss has not been reported. It might be that loss of PTEN expression can only partially be explained by down regulation via miRs.

A downstream component of the PI3K/AKT pathway, known as the mTOR pathway (mammalian Target of Rapamycin), is up regulated in many cancers. As a consequence of PTEN loss, activation of the mTOR pathway may occur. As shown in, *in vitro* and *in vivo* experiments, cells without PTEN are more sensitive to mTOR inhibitors (rapamycin) (32,33). These mTOR inhibitors might provide efficacious additional therapy in cervical cancer patients with an unfavourable prognosis and loss of PTEN.

Our study indicates a strong relation between squamous cell carcinomas and EGFR staining, which was previously reported in cervical cancer by Kersemaekers *et al.* (11). In their study, EGFR overexpression was observed in 54% (73/136) cervical cancer cases and was associated with reduced disease-free and reduced overall survival in surgically treated cervical cancer patients (11). These findings could not be confirmed in our large series of cervical cancer patients, where 32.1% of cases overexpressed EGFR. One explanation might be the use of a different antibody. Another possibility is that our study included a larger percentage of adenocarcinomas (28% vs. 9%) and as is shown in our study, EGFR expression is highly associated with squamous cell carcinoma.

In conclusion, in early stage cervical cancer, loss of PTEN expression is associated with pelvic lymph node metastasis, suggesting that PTEN is one of the tumor suppressor genes affecting pelvic lymph node metastasis in early stage cervical cancer. Overall however, the EGFR pathway does not appear to have prognostic impact in surgically treated early stage cervical cancer.

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Involvement of the TGF-β and β-catenin pathways in pelvic lymph node metastasis in early stage cervical cancer

Submitted for publication

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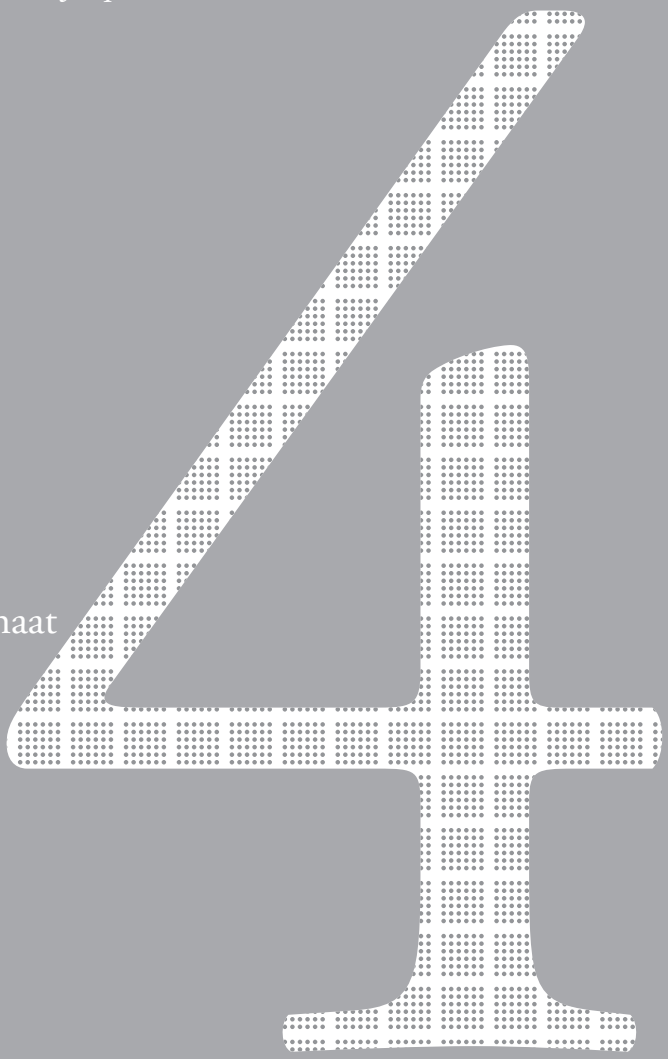

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Abstract

Presence of pelvic lymph node metastases is the main prognostic factor in early stage cervical cancer patients, primarily treated with surgery. The aim of this study was to identify cellular tumor pathways associated with pelvic lymph node metastasis in early stage cervical cancer. Gene expression profiles (Affymetrix U133 plus 2.0) of 20 patients with negative (N_0) and 19 with positive lymph nodes (N+), were compared with gene sets that represent well-known and novel pathway signatures (n=285). Differentially expressed genes were identified using a random-variance t-test. Pathway analysis showed significant enrichment of the TGF-β pathway in N_0 patients, while dysregulation of the β-catenin pathway was associated with N+. Of the most significant 149 genes that were differentially expressed between N_0 and N+ tumors (P<0.001), five genes were involved in β-catenin signaling (*TCF4*, *CTNNAL1*, *CTNND1/p120*, *DKK3* and *WNT5a*). Validation by immunostaining of tumors of 274 consecutive early stage cervical cancer patients was performed for representatives of the identified pathways. This analysis confirmed that positive immunostaining of Smad4 (TGF-β pathway) was related to N_0 (OR=0.20; 95%CI=0.06 – 0.66) and p120 positivity (β-catenin pathway) to N+ (OR=1.79; 95%CI=1.05 – 3.05). In conclusion, our study provides new insights in the molecular mechanism of lymph node metastasis in cervical cancer. Pathway analysis of the microarray expression profile revealed that the TGF-β and p120-associated β-catenin pathways are important in pelvic lymph node metastasis in early stage cervical cancer.

Introduction

Standard treatment of early stage cervical cancer patients consists of radical hysterectomy and pelvic lymphadenectomy. For this group of patients, the presence of lymph node metastases is the most important prognostic factor (1). Early stage cervical cancer patients with negative lymph nodes have a 5-year survival of 90% vs. only 65% in patients with lymph node metastases (2). Patients with lymph node metastases are therefore treated with adjuvant (chemo)radiation. However, the combination of surgery and (chemo)radiation is associated with severe morbidity (3). If the presence of metastatic lymph nodes could be predicted prior to treatment, primary chemoradiation could be considered, which is equally effective, but associated with a different treatment-related morbidity pattern.

Several histopathological characteristics such as tumor size, lymph vascular space involvement and depth of invasion have been associated with lymph node metastases in cervical cancer but none of these is of sufficient clinical relevance (4). Furthermore, various molecular tumor markers like the expression of vascular endothelial growth factor (VEGF), and p16 have been reported to be related with lymph node metastases in cervical cancer (5,6), but presently no markers are available to predict lymph node status with high sensitivity and specificity. Non- and minimal invasive diagnostic techniques, such as sentinel lymph node biopsy are currently being explored to better identify patients with disease outside the cervix (7).

Little is known about biological pathways involved in lymph node metastasis in cervical cancer. Metastasis is a complex, multistep process involving decreased cell-cell interaction, increased cell migration, disruption of the basal membrane, intravasation into the circulation, survival of direct exposure to the immune system and extreme mechanic forces in the bloodstream, and finally extravasation and growth in metastatic sites (8). Apart from tumor-specific changes, many processes in the tumor microenvironment of the primary tumor have shown also to be important for initiation of the metastatic potential at the primary site (9).

Gene expression profiling has provided tools to identify patterns of biological differences between different tumor types, cancers with diverse clinical outcome or treatment responses (10,11). To get insight into the mechanism of lymph node metastasis in head and neck (12), colorectal (13), and cervical cancer (14-17), gene expression profiling has been used. However, in most studies little overlap was found between differentially expressed genes, which may be due to a variety of methodological issues (18). Explanations that have been debated extensively in the literature are the use of different microarray platforms (18,19) and the restricted number of samples used to select genes

from a large pool of probes (20). Therefore, comparing gene expression profiles with gene sets that represent unique pathways may provide more insight into the mechanism of lymph node metastasis. Different pathway analysis methods have been developed, including Gene Set Enrichment Analysis (GSEA). GSEA is used to determine whether pre-defined gene sets available for example in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (21) and Biocarta data bases (http://www.biocarta.com/), show significant, concordant differences between two phenotypes (22). Another method has recently been developed by Bild *et al.* (23). Experimentally generated expression signatures using human primary mammary epithelial cell cultures (HMECs) that reflect the activation of various oncogenic signaling pathways (c-Myc, H-Ras, c-Src, E2F3, and β-catenin) can be used to assess the activation probability of the oncogenic pathways in individual expression profiles. Both methods have not been applied previously for differentiating between lymph node negative and positive cervical cancer patients.

The aim of this study was to identify cellular tumor pathways associated with pelvic lymph node status in patients with early stage cervical cancer. Apart from obtaining more insights on the molecular processes of lymph node metastasis in early stage cervical cancer, our findings might contribute to individual treatment strategies. To identify such pathways, expression array analysis was performed on a well defined series of cervical squamous cell carcinomas of patients with histologically confirmed lymph node metastases (N+) vs. patients with histological and clinically confirmed negative lymph nodes (N_0). Potential markers representing the predictive value of pathways were validated in a large consecutive series of early stage cervical cancer patients by immunohistochemistry on tissue microarrays (TMA).

Materials and methods

Patients and tumor samples

Since 1980 clinicopathological characteristics of all cervical cancer patients referred to the Department of Gynecological Oncology of the University Medical Center Groningen are prospectively collected in a database. For the present study, patients with stage IB-IIA disease, primarily treated with surgery between 1980 and 2004 were selected (n=337). Follow-up data were collected for at least five years. Staging was performed according to FIGO guidelines. Primary treatment consisted of type 3 radical hysterectomy and pelvic lymph node dissection. In case of poor prognostic factors, such as lymph node metastases or positive resection margins, patients were treated with adjuvant radiotherapy or chemoradiation. From these patients paraffin-embedded formalin-

fixed primary tumor tissue was collected. All tumor tissues were histological revised and only tumor specimens with sufficient tumor cells were included in the study for construction of the TMA. In 274 cases sufficient pre-treatment paraffin-embedded tissue was available for TMA construction. 112/274 (41%) patients received adjuvant (chemo)radiation. Median follow-up time for patients on the TMA was 5.5 years (range 0.3 – 18.6). Since 1990, when sufficient material was available also pre-treatment fresh frozen tumor tissue was stored. For the microarray experiment, we selected fresh frozen primary cervical cancer tissue, containing at least 80% tumor cells, of patients with histologically confirmed N_0 (n=20) and of patients with N+ (n=19). The N_0 and N+ groups were matched for age, FIGO stage and histology (all squamous cell carcinoma). However, as expected the groups differed regarding presence of lymphangioinvasion (P=0.024) and infiltration depth (P=0.001). Patient and tumor characteristics are summarized in Table 1. In the University Medical Center Groningen clinicopathologic and follow-up data are prospectively obtained during standard treatment and follow-up and stored in a computerized registration database. For the present study, all relevant data were retrieved from this computerized database into a separate, anonymous database. Patient identity was protected by study-specific, unique patient numbers. Codes were only known to two dedicated data managers, who also have daily responsibility for the larger database. In case of uncertainties with respect to clinicopathologic and follow-up data, the larger databases could only be checked through the datamanagers, thereby ascertaining the protection of patients' identity. Using the registration database all tissue specimens were identified by unique patient numbers and retrieved from the archives of the Department of Pathology. Therefore, according to Dutch law no further Institutional Review Board approval was required (http://www.federa.org/).

Microarray experiments

From the frozen biopsies, four 10-μm-thick sections were cut and used for standard RNA isolation. After cutting, a 3-μm-thick section was stained with hematoxylin/eosin for histological examination and only tissues with >80% tumor cells were included. RNA was isolated with TRIzol reagent (Invitrogen, Breda, the Netherlands) according to manufacturer's protocol. RNA was treated with DNAse and purified using the RNeasy mini-kit (Qiagen, Westburg, Leusden, the Netherlands). The quality and quantity of the RNA was determined by Agilent Lab-on-Chip analysis. For labelling, 10 μg of total RNA was amplified by *in vitro* transcription using T7 RNA polymerase. Labelled RNA samples were hybridized according to a randomized design to the human genome U133 plus 2.0 microarrays (Affymetrix, Santa Clara, CA). The microarrays were loaded

Table 1 – Patient and tumor characteristics

	Microarray N_0 * n=20		Microarray N+ † n=19		TMA‡ n=274	
Age at diagnosis						
Median	47.39		40.44		43.65	
Range	31.53 – 72.71		29.10 – 72.51		23.67 – 84.65	
	n	*%*	*n*	*%*	*n*	*%*
FIGO stage						
Ib1	11	55%	10	53%	174	64%
Ib2	5	25%	6	32%	54	20%
IIa	4	20%	3	16%	46	17%
Histology						
Squamous cell carcinoma	20	100%	19	100%	182	66%
Adenocarcinoma	0	0%	0	0%	74	27%
Other	0	0%	0	0%	18	7%
Grade of differentiation						
Good/moderate	15	75%	10	53%	163	59%
Poor/undifferentiated	4	20%	9	47%	106	39%
unknown	*1*	*5%*	*0*	*0%*	*5*	*2%*
Lymphangioinvasion						
No	14	70%	6	32%	132	48%
Yes	6	30%	12	63%	142	52%
unknown	*0*	*0%*	*1*	*5%*	*0*	*0%*
Infiltration depth						
0-10 mm	14	70%	3	16%	135	49%
≥10 mm	5	25%	14	74%	126	46%
unknown	*1*	*5%*	*2*	*11%*	*13*	*5%*
Tumor diameter						
0-4 cm	14	70%	12	63%	198	72%
≥4 cm	6	30%	7	37%	76	28%
Lymph nodes						
Negative	20	100%	0	0%	194	71%
Positive	0	0%	19	100%	80	29%

*Lymph node negative
†Lymph node positive
‡Tissue microarray

with 200 μl of hybridization cocktail solution and then placed in Genechip Hybridization Oven 640 (Affymetrix) rotating at 60 rpm at 45 °C for 16 h. After hybridization, the arrays were washed on Genechip Fluidics Station 400 (Affymetrix) and scanned using Genechip Scanner 3000 (Affymetrix) according to the manufacturers' procedure. Labeling of the RNA, quality control, the microarray hybridization and scanning were

performed by ServiceXS (Leiden, the Netherlands, http://www.serviceXS.com) according to Affymetrix standards. Pre-processing of CEL files was performed with Affymetrix Expression Console software. Probe set expression summary was done using the Robust Multi-array Average (RMA) algorithm. Quality of the microarray data was checked using histograms, box plots and a RNA degradation plot. Principal component analysis (PCA) was performed for controlling the quality of the hybridizations (24).

Gene Set Enrichment Analysis (GSEA)

GSEA was performed with the software package GSEA 2.0, developed by the Broad Institute of MIT and Harvard (22). Each gene was ranked according to its relative difference in expression between the N_0 and N+ group using the Student's t-statistic. Ranked expression data for all annotated 20,606 genes (in case of more than one probe per gene, the probe with the highest intensity was considered) were compared against a large collection of biological gene sets to determine whether genes both at the top or bottom of the ranked list were enriched in these functional gene sets. GSEA analysis was performed separately with a total of 155 gene sets in the KEGG (21) and 125 gene sets in the Biocarta data base. The gene sets used are available at the Molecular Signature Database (http://www.broadinstitute.org/gsea/msigdb/). Statistical enrichment was determined using an empirical phenotype-based permutation test based on 1,000 permutations. Furthermore, for each functional set the false discovery rate (FDR) and nominal *P* value were calculated. *P* values of <0.05 were considered statistically significant.

Oncogenic pathway activation

Bild *et al.* experimentally generated expression signatures using human primary mammary epithelial cell cultures (HMECs) that reflect the activation of various oncogenic signaling pathways (c-Myc, H-Ras, c-Src, E2F3, and β-catenin) (23). Recombinant adenoviruses were used to express the oncogenic activity in an otherwise quiescent cell. Genes differently expressed between the quiescent cells and the transfected cells were selected and were used in a model to predict the activation status of each of these five oncogenic pathways. Publicly available software implementing these models (BinReg) (23) was used to assess the activation probability of the oncogenic pathways in our 39 cervical tumor samples. In this analysis, it was expected to find a large difference in expression patterns between the HMECs Bild *et al.* used to generate the oncogenic expression signatures and our cervical tumor samples. The model to predict the activation

status is based solely on the expression data of the HMECs and does not take into account the expression differences between the HMECs and the cervical tumor samples. This could potentially lead to unreliable activation probabilities. Therefore, the HMECs and cervical tumor samples were pooled and by applying Principle Component Analysis (PCA) we aimed to identify a Principal Component (PC) that explained variance correlating with the difference between HMECs and cervical tumor samples (25,26). By subtracting the variance explained by this PC it is possible to filter out the expression differences between HMECs and cervical tumor samples. Therefore, the variance explained by this PC was subtracted from our data set and these corrected cervical tumor data was used for subsequent analysis with BinReg.

Class comparison

Class comparison was performed using the software package BRB Array Tools 3.7.0, developed by the Biometric Research Branch of the US National Cancer Institute (http://linus.nci.nih.gov/BRB-ArrayTools.html). Differentially expressed probe sets were identified using a parametric two-sample t-test (with random variance model) with a significance threshold of P<0.001. In addition, for each probe set the FDR was determined (27). Finally, a global test was performed to assess the probability of getting the observed number of identified significant probe sets by chance, that is, under the assumption that there is no difference in expression between the N_0 and N+ group. Differentially expressed genes were ranked according to lowest FDR and lowest parametric P value.

Immunohistochemical validation

Immunohistochemistry of the relevant proteins was performed on tissue microarrays (TMAs). TMAs were constructed as previously described (28). For immunohistochemistry, 3 μm sections were cut from the TMAs. These sections were mounted on amino-propyl-ethoxy-silan (APES, Sigma-Aldrich, Diesenhofen Germany)-coated glass slides. Slides were deparaffinized in xylene and rehydrated in ethanol. Antigen retrieval was performed by boiling the slides in a microwave oven in citrate (pH 6.0) for 15 min. Endogenous peroxidase was blocked by incubation with 0.3% hydrogen peroxidase for 30 min. Slides were incubated with a mouse monoclonal antibodies against β-catenin (clone 14; dilution 1:1000; BD Transduction Laboratories, Franklin Lake, NJ) and p120 (clone 98; dilution 1:100; BD Transduction Laboratories) for one hour at room temperature, and against Smad4 (clone B-8; dilution

1:400; Santa Cruz Biotechnology, Santa Cruz, CA) and pSmad2 (clone 138D4; dilution 1:25; Cell Signaling, Danvers, MA) overnight at 4ºC. For immunodetection of β-catenin and Smad4, RAMHRP (dilution 1:100; Dako, Copenhagen, Denmark) and GARHRP(dilution 1:100; Dako) were used. For immunodetection of pSmad2 GARHRP (dilution 1:100; Dako) and RAGHRP(dilution 1:100; Dako) and for p120 the EnVision horseradish peroxidase system (Dako) were used. Staining was visualized by 3'3-diaminobenzidinetetrahydrochloride and counterstaining was performed with hematoxylin. Normal cervical epithelium was used as a positive control. Scoring was performed by two independent observers without knowledge of clinical data. A concordance of more than 90% was found for all stainings. The discordant cases were reviewed and scores were reassigned on consensus of opinion. Staining intensity was semiquantitatively scored as negative (0), weak positive (1), moderate positive (2), and strong positive (3). Also the percentage of positive cells was recorded. Positive Smad4 expression was defined as presence of both >50% moderate/strong positive nuclear and moderate/strong positive cytoplasmic staining (29). β-catenin and p120 positivity was defined as membranous staining at any intensity (1-3) in >50% of cells (30). Statistical analysis was performed with SPSS 16.0 for Windows (SPSS Inc., Chicago, IL). Associations between immunostainings and lymph node metastases were compared using logistic regression models, in which immunostainings were used as dependent factors and the clinicopathological characteristics as independent factors. *P* values of <0.05 were considered statistically significant.

Table 2 – Results of Gene Set Enrichment Analysis using pathway definitions of Biocarta and KEGG

Pathway	*P* value	FDR‡	Enriched in
NFAT (Biocarta)	0.004	0.252	N_0*
ALK (Biocarta)	0.013	0.269	N_0*
BAD (Biocarta)	0.016	0.492	N_0*
TGF-β (KEGG)	0.027	1.000	N_0*
Glycosphingolipid Biosynthesis Neo Lactoseries (KEGG)	0.039	1.000	N+†
PAR1 (Biocarta)	0.046	0.907	N_0*

*Lymph node negative
†Lymph node positive
‡False discovery rate

Table 3 – Predicted probabilities for all 5 oncogenic pathways

Lymph node negative

*CXCA**	*β-catenin*	*H-Ras*	*c-Src*	*c-Myc*	*E2F3*
12	0.387	0.868	0.635	0.690	0.287
07	0.792	0.278	0.655	0.702	0.562
11	0.249	0.593	0.487	0.596	0.738
20	0.647	0.180	0.539	0.485	0.572
08	0.331	0.331	0.352	0.704	0.952
10	0.368	0.715	0.500	0.536	0.434
13	0.793	0.129	0.718	0.304	0.366
19	0.539	0.442	0.625	0.590	0.334
14	0.625	0.278	0.733	0.499	0.121
18	0.552	0.557	0.555	0.428	0.296
16	0.287	0.512	0.275	0.826	0.982
15	0.465	0.513	0.528	0.566	0.238
17	0.406	0.485	0.526	0.582	0.164
09	0.256	0.601	0.448	0.370	0.351
02	0.427	0.410	0.478	0.474	0.783
05	0.594	0.200	0.458	0.475	0.850
03	0.498	0.557	0.592	0.344	0.158
06	0.384	0.482	0.425	0.392	0.488
04	0.542	0.584	0.516	0.583	0.812
01	0.535	0.338	0.497	0.443	0.503

Lymph node positive

*CXCA**	*β-catenin*	*H-Ras*	*c-Src*	*c-Myc*	*E2F3*
23	0.673	0.299	0.601	0.583	0.808
22	0.750	0.208	0.704	0.452	0.288
36	0.535	0.439	0.539	0.438	0.328
39	0.686	0.251	0.614	0.417	0.240
21	0.608	0.550	0.530	0.360	0.682
38	0.518	0.475	0.454	0.700	0.405
35	0.487	0.372	0.523	0.636	0.540
37	0.572	0.615	0.574	0.514	0.447
24	0.584	0.573	0.626	0.574	0.371
34	0.901	0.180	0.858	0.339	0.167
27	0.570	0.762	0.615	0.597	0.312
29	0.579	0.386	0.601	0.519	0.558
30	0.539	0.488	0.562	0.372	0.235
31	0.708	0.199	0.492	0.486	0.598
28	0.724	0.283	0.671	0.418	0.440
26	0.620	0.527	0.601	0.483	0.400
25	0.654	0.257	0.626	0.441	0.459
32	0.724	0.122	0.699	0.344	0.216
33	0.705	0.246	0.596	0.609	0.278

*Cervical cancer ID

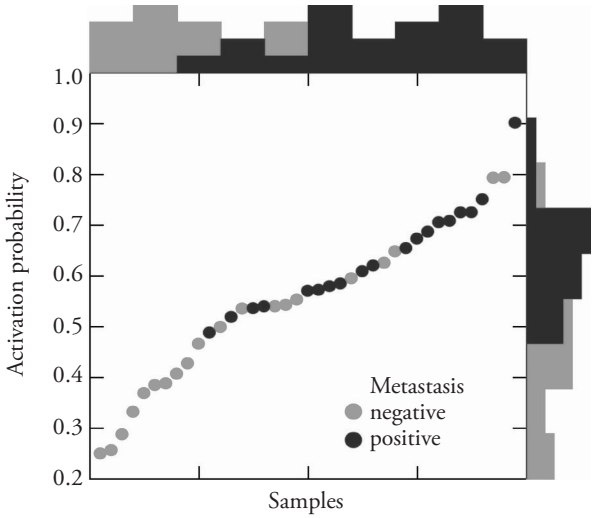
Results

Biological pathways associated with pelvic lymph node status

GSEA using biological pathway definitions according to KEGG and Biocarta data bases revealed that five pathways (TGF-β, NFAT, ALK, BAD and PAR1 pathway) were significantly enriched in the N_0 group, whereas only one pathway (Glycosphingolipid Biosynthesis Neo Lactoseries pathway) was enriched in the N+ group (Table 2).

Analyzing the association between oncogenic pathways and lymph node status using expression signatures that reflect the activation of five major oncogenic signaling pathways (c-Myc, H-Ras, c-Src, E2F3, and β-catenin) revealed that the activation probabilities of the oncogenic β-catenin pathway correlated highly significantly with N+ (P=0.001). Table 3 shows the predicted probabilities for all five oncogenic pathways. A scatter plot of the activation probability of β-catenin for our 39 cervical tumor samples shows that tumor samples with a low or high probability of β-catenin activation are predominantly N_0 or N+ tumor samples, respectively (Fig. 1).

Figure 1 – Scatter plot of the activation probability of β-catenin for the 39 cervical tumor samples



Individual genes of the β-catenin pathway are related to lymph node status

We identified probe sets that were differentially expressed between N_0 and N+ samples using a random-variance t-test. Using this analysis, we identified 188 probe sets that are differentially expressed at a significance level of $P<0.001$ (Table 5). The probability of finding at least 188 significant probe sets by chance, that is, under the assumption that there are no differences between the N_0 and N+ groups was $P=0.035$. These 188 probe sets represented 149 unique genes of which 46 genes were upregulated and 103 genes were downregulated in the N+ group. Interestingly, 14 probe sets representing five unique genes (*TCF4*, *CTNNAL1*, *DKK3*, *CTNND1/p120* and *WNT5a*) belong to the β-catenin pathway. This is in good agreement with our pathway analysis using all genes.

Immunohistochemical validation of the TGF-β and β-catenin pathway

To validate the association between the lymph node status in early stage cervical cancer and the oncogenic TGF-β signaling and β-catenin pathways, we performed immunohistochemistry using antibodies directed against proteins that are representative for both these pathways. For this purpose, we used a series of pre-treatment early stage cervical

cancer tissues of 274 patients.

Phosphorylation of Smad2/3 and concomitant translocation into the nucleus is an important step in transforming growth factor β (TGF-β) signaling and expression of Smad4 is an essential partner of Smad2/3 in the formation of transcriptional complexes (31,32). To validate whether pSmad2 and/or Smad4 staining on the TMA are representative for the whole tumor, first whole tumor slides of a small series of 20 randomly selected cervical cancer tissues were immunostained. This immunostaining revealed that only Smad4 staining was homogeneous (data not shown). Therefore, Smad4 staining on the TMA reflects best the staining of the whole tumor. Thirty five out of 255 evaluable cervical carcinomas showed positive Smad4 staining (see Fig. 2 for representative immunostainings). Univariate logistic regression analysis of various clinicopathological features revealed that Smad4 positivity was not only related to N_0, (OR=0.20; 95%CI=0.06 – 0.66) but also to infiltration depth <10 mm (OR=0.35; 95%CI=0.16 – 0.76) (Table 4).

To validate whether β-catenin signaling is associated with the presence of lymph node metastases in cervical cancer, immunohistochemical staining was performed for β-catenin, a key protein in the canonical β-catenin pathway (33). In addition, we included the immunostaining for CTNND1/p120 that is involved in non-canonical β-catenin signaling (34) and was one of the five β-catenin related transcripts present in the list of 149 differentially expressed genes (188 probe sets) (Table 5). Positive p120 immunostaining was observed in 112/268 (42%) and positive β-catenin in 140/272 (51%) patients (see Fig. 2 for examples). Logistic regression analysis revealed no association between β-catenin protein expression and presence of lymph node metastases (Table 4). However, positive p120 staining was associated with N+ (OR=1.79; 95%CI=1.05 – 3.05), in agreement with our microarray results.

Discussion

In the present study, pathways associated with pelvic lymph node metastases in 39 (20 N_0 and 19 N+) early stage cervical cancer patients were identified. Our analysis of well-known and novel (n=285) pathway signatures revealed an association of lymph node metastases with only few gene sets or signatures, including two well-known oncogenic biological gene sets. Enrichment of the TGF-β pathway was related to N_0, while oncogenic pathway activation of β-catenin was associated with N+ patients. The association of both the TGF-β and the β-catenin signaling pathway with lymph node metastases was validated in a large consecutive series of early stage cervical cancer patients by im-

Table 4 – Logistic regression analysis for the relation between clinicopathological characteristics and stainings

Smad4 (n=255)	**Smad4 -**		**Smad4 +**		**Smad4 +**	
	n/total	*%*	*n/total*	*%*	*OR* †	*(95%CI)* ‡
Age (continuous)					1.00	(0.98 – 1.03)
Age ≥43	111/220	50%	21/35	60%		
Stage ≥Ib2	83/220	38%	11/35	31%	0.76	(0.35 – 1.62)
SCC*	150/206	73%	20/31	65%	0.68	(0.31 – 1.51)
Poor differentiation	87/216	40%	17/34	50%	1.48	(0.72 – 3.06)
Lymphangioinvasion	119/220	54%	15/35	43%	0.64	(0.31 – 1.31)
Infiltration depth ≥10 mm	111/207	54%	10/35	29%	**0.35**	**(0.16 – 0.76)**§
Tumor diameter ≥4 cm	65/220	30%	6/35	17%	0.49	(0.20 – 1.24)
Positive lymph nodes	71/220	32%	3/35	9%	**0.20**	**(0.06 – 0.66)**§
p120 (n=268)	**p120 -**		**p120 +**		**p120 +**	
	n/total	*%*	*n/total*	*%*	*OR* †	*(95%CI)* ‡
Age (continuous)					1.00	(0.98 – 1.02)
Age ≥43	78/156	50%	60/112	54%		
Stage ≥Ib2	58/156	37%	40/112	36%	0.94	(0.57 – 1.56)
SCC*	88/142	62%	90/108	83%	**3.07**	**(1.67 – 5.64)**§
Poor differentiation	64/153	42%	41/110	37%	0.83	(0.50 – 1.37)
Lymphangioinvasion	70/156	45%	68/112	61%	**1.90**	**(1.16 – 3.11)**§
Infiltration depth ≥10 mm	70/147	48%	54/108	50%	1.10	(0.67 – 1.81)
Tumor diameter ≥4 cm	44/156	28%	31/112	28%	0.97	(0.57 – 1.67)
Positive lymph nodes	37/156	24%	40/112	36%	**1.79**	**(1.05 – 3.05)**§
***β*-catenin (n=272)**	***β*-catenin -**		***β*-catenin +**		***β*-catenin +**	
	n/total	*%*	*n/total*	*%*	*OR* †	*(95%CI)* ‡
Age (continuous)					1.01	(0.99 – 1.03)
Age ≥43	63/132	48%	76/140	54%		
Stage ≥Ib2	48/132	36%	52/140	37%	1.03	(0.63 – 1.69)
SCC*	81/126	64%	101/129	78%	**2.00**	**(1.15 – 3.49)**§
Poor differentiation	52/132	39%	53/135	39%	0.99	(0.61 – 1.62)
Lymphangioinvasion	63/132	48%	77/140	55%	1.34	(0.83 – 2.16)
Infiltration depth ≥10 mm	64/125	51%	61/134	46%	0.80	(0.49 – 1.30)
Tumor diameter ≥4 cm	38/132	29%	38/140	27%	0.92	(0.54 – 1.57)
Positive lymph nodes	37/132	28%	43/140	31%	1.14	(0.67 – 1.92)

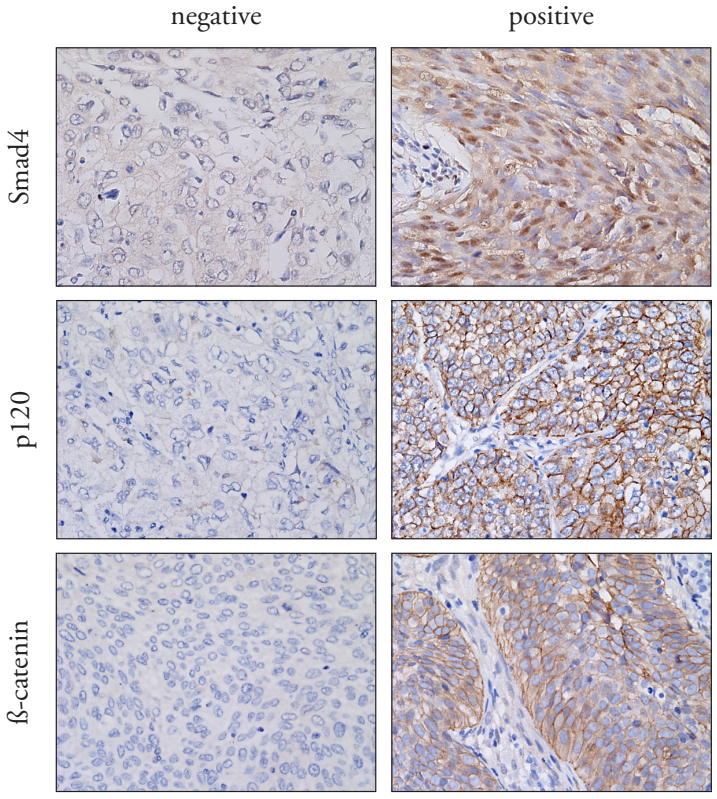
The proportion of patients with less than 2 representative tissue cores varied from 1-7%

*Squamous cell carcinoma
†Odds ratio
‡95% confidence interval
§*P* value <0.05

munohistochemistry. Immunostaining of Smad4 and p120 representing the TGF-*β* and *β*-catenin signaling pathway, respectively, confirmed the association with lymph node metastasis in early stage cervical cancer.

Until now, all studies using microarray platforms for differentiating between patient with and without lymph node metastases in cervical cancer focused on gene profiles

Figure 2 – Representative immunostaining patterns for Smad4, p120, and β-catenin at 400x magnification.



and individual genes present in these profiles (14-17). Another approach is to identify biological pathways that are involved in biological differences between cancers, using pathway analysis methods on all genes that are differentially expressed between two phenotypes. For example, Lagarde *et al.* identified pathways that differentiated between N_0 and N+ esophageal adenocarcinomas (35). Furthermore, Crijns *et al.* identified pathways contributing to clinical outcome of serous ovarian cancer (24). Interestingly, many of these pathways were known for being important in carcinogenesis or cancer progression, which indicates the strength of this approach. To our knowledge, we are the first to identify pathways for discriminating between N_0 and N+ cervical cancer patients using pathway analysis methods.

Our analysis showed that TGF-β is one of the most important pathways affecting the metastatic potential in early stage cervical cancer. First, of all 280 tested unique pathways (from the KEGG and Biocarta data bases), the TGF-β pathway was signifi-

cantly enriched in N_0 (Table 2). Binding of the TGF-β ligand to its receptors initiates intracellular signaling by phosphorylation of Smad2 and Smad3. These phosphorylated Smads then bind to Smad4 and translocate into the nucleus, where this Smad complex is involved in regulation of gene transcription (31,32). Immunostaining using Smad4 of 255 early stage cervical carcinomas confirmed that TGF-β pathway activation was related to absence of lymph node metastases. Early in carcinogenesis, the TGF-β pathway contributes to tumor suppression, for example by stimulating apoptosis and inhibition of growth (31,32) However, later in the process of tumor progression or in invasive cancer, oncogenic activity of TGF-β signaling is predominantly present, including increased migration and invasiveness, which may result in metastases. This transition from a tumor suppressor to an oncogenic pathway can be due to various alterations in TGF-β signaling, such as loss of Smad signaling and activation of Smad-independent, more oncogenic pathways, such as MAPK pathways (31,32). Furthermore, TGF-β is directly involved in the formation of metastases, as it contributes to the establishment and outgrowth of lung and bone metastases in breast cancer models (36,37). Smad4 downregulation is associated with TGF-β downregulation and has been implicated in cervical cancer (29) and metastatic mouse models (36). The downregulation of Smad4 in N+ is consistent with these data and establishes TGF-β as one of the pathways affecting the metastatic potential in early stage cervical cancer.

In addition to the TGF-β pathway, GSEA revealed that the NFAT, ALK, BAD, and PAR1 pathways were significantly enriched in the N_0 group and the Glycosphingolipid Biosynthesis Neo Lactoseries pathway in the N+ group (Table 2). Presently, little is known about these pathways and whether they are associated with the metastatic behavior of tumor cells. The elucidation of the possible involvement of these pathways in lymph node metastasis is subject of future interest in our laboratory.

A limitation of GSEA is that pathway activation can not be assessed for an individual patient. Therefore, another strategy was developed in which expression signatures are experimentally generated to reflect activation status of various oncogenic signaling pathways (23). Our study indicates that N+ patients had a higher probability of β-catenin pathway activation than N_0 patients, pointing to a role for the β-catenin pathway in formation of lymph node metastases. Interestingly, the gene set of 188 differentially expressed probe sets between N_0 and N+, included five unique genes involved in the β-catenin-pathway including *p120 (CTNND1* or *catenin delta 1*), *CTNNAL1* (*catenin alpha-like 1*), *DKK3* (*dickkopf homolog 3*), *WNT5a*, and *TCF4* (*transcription factor 4*), but did not include β-catenin. In good agreement with these findings, immunohistochemistry confirmed the association of p120 and the lack of correlation of β-catenin

with N+. β-catenin is an important member of both the WNT-signaling pathway and the cell-cell adhesion pathway. However, immunohistochemical analysis revealed no relation between β-catenin and lymph node metastases, which is in agreement with other studies (30,38) and indicates that the canonical Wnt/β-catenin pathway (containing β-catenin, Wnt1, APC) is not involved in mediating the invasive potential in cervical cancer. In normal cervical epithelium, β-catenin is involved in E-cadherin mediated cell-cell adhesion, by binding to the cytoplasmic domain of E-cadherin. Loss of E-cadherin causes disruption of cell adhesion and therefore might contribute to metastases (33,34). P120 (also referred to as CTNND1 or delta-catenin) is a member of the catenin family and was originally reported to stabilize the cadherin-complex by direct interaction with the proximal domain of E-cadherin (33,34). On the other hand, p120 (especially p120 isoform 1) promotes cell motility and invasiveness in cancer (39). P120 was reported to exert its effects by modulating the activities of Rho GTPases, for example by inhibiting activity of RhoA and activation of Rac and Cdc42 (39,40). To our knowledge our study is the first that reports that p120 expression is associated with presence of lymph node metastases in early stage cervical cancer. The link of p120 to Rho GTPases in activating the metastatic potential might also offer new opportunities for therapy since invasion has been inhibited successfully using Rho-inhibitors (41).

Thus, both the TGF-β and the β-catenin pathway are related to lymph node metastases in cervical cancer. This indicates an important role for epithelial to mesenchymal transition (EMT), as both pathways may contribute to EMT. EMT is characterized by loss of the epithelial phenotype of cells and cells adopt a mesenchymal phenotype. It can be induced by alterations in TGF-β signaling, such as loss of Smad4 (42) and EMT is characterized by loss of E-cadherin, with disruption of cell adhesion as a consequence. Furthermore, EMT results in increased motility of cells, and increased invasion. All these processes contribute to the formation of metastases (43,44). TGF-β signaling and β-catenin also cooperate in EMT. Loss of E-cadherin causes increased β-catenin signaling, which cooperates with autocrine TGF-β signaling to maintain an mesenchymal phenotype (45). Thus, deregulation of both the TGF-β and the β-catenin pathway, as observed in our study, indicates a role for EMT in lymph node metastasis in cervical cancer. Interestingly, miR-200a which is known for inhibition of TGF-β-mediated EMT by maintaining the epithelial phenotype through regulating expression of the E-cadherin transcriptional repressors ZEB1 and ZEB2 (46), was found to be a suppressor of metastasis in cervical cancer (47). This supports the importance of EMT in lymph node metastasis in cervical cancer.

Presence of lymph node metastases is still one of the most important factors in the

choice of treatment for early stage cervical cancer patients. No markers are currently available for accurate prediction of lymph node metastases before primary surgery. Expression levels of proteins such as Smad4 and p120 as representatives for the TGF-β signaling and β-catenin pathway respectively, can also not accurately predict lymph node metastases. However, more detailed analysis of these pathways might result in the identification of additional markers that will increase the clinical sensitivity en specificity. More importantly, by identifying pathways involved in lymph node metastasis in early stage cervical cancer, new opportunities for pathway targeted therapy can be considered to inhibit the metastatic potential, as reported for both pathways (48,49).

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Table 5 – 188 probe sets differentially expressed between N_0 samples and N+ samples

Upregulated in N_0

Rank	*Parametric P value*	*FDR*	*Fold-change*	*Probe set*	*Gene symbol*	*Description*
1	0.0000012	0.042	2.054	222146_s_at	TCF4	transcription factor 4
3	0.0000023	0.042	1.623	209250_at	DEGS1	degenerative spermatocyte homolog 1, lipid desaturase (Drosophila)
4	0.0000064	0.075	1.795	212387_at	TCF4	transcription factor 4
5	0.0000069	0.075	2.173	203753_at	TCF4	transcription factor 4
7	0.0000142	0.111	2.165	226931_at	TMTC1	transmembrane and tetratricopeptide repeat containing 1
8	0.0000168	0.115	1.979	212382_at	TCF4	transcription factor 4
9	0.0000197	0.120	1.684	232304_at	PELI1	pellino homolog 1 (Drosophila)
10	0.0000221	0.121	1.391	1559249_at	ATXN1	ataxin 1
11	0.0000264	0.125	1.556	209281_s_at	ATP2B1	ATPase, Ca++ transporting, plasma membrane 1
12	0.0000318	0.125	1.473	221683_s_at	CEP290	centrosomal protein 290kDa
13	0.0000323	0.125	2.795	226084_at	MAP1B	microtubule-associated protein 1B
15	0.0000344	0.125	1.504	212509_s_at	MXRA7	matrix-remodelling associated 7
16	0.0000414	0.127	1.807	214724_at	DIXDC1	DIX domain containing 1
17	0.0000416	0.127	1.818	212386_at	TCF4	transcription factor 4
18	0.0000429	0.127	1.791	213891_s_at	TCF4	transcription factor 4
19	0.0000445	0.127	1.571	226546_at		Not available
20	0.0000483	0.127	1.672	226676_at	ZNF521	zinc finger protein 521
21	0.0000495	0.127	2.243	227812_at	TNFRSF19	tumor necrosis factor receptor superfamily, member 19
22	0.0000513	0.127	2.269	226322_at	TMTC1	transmembrane and tetratricopeptide repeat containing 1
23	0.0000562	0.134	2.047	225946_at	RASSF8	Ras association (RalGDS/AF-6) domain family 8
24	0.0000627	0.141	1.528	235834_at	CALD1	caldesmon 1
25	0.0000655	0.141	1.786	1554007_at	ZNF483	zinc finger protein 483
26	0.0000677	0.141	1.668	231869_at	ZNF451	zinc finger protein 451
28	0.0000738	0.141	1.369	207604_s_at	SLC4A7	solute carrier family 4, sodium bicarbonate cotransporter, member 7
29	0.0000748	0.141	2.051	235599_at	LOC339535	hypothetical protein LOC339535
30	0.0000792	0.143	1.382	202126_at	PRPF4B	PRP4 pre-mRNA processing factor 4 homolog B (yeast)
31	0.0000810	0.143	1.704	235592_at	ELL2	elongation factor, RNA polymerase II, 2
32	0.0000849	0.145	1.516	208662_s_at	TTC3	tetratricopeptide repeat domain 3
34	0.0000913	0.147	1.674	209682_at	CBLB	Cas-Br-M (murine) ecotropic retroviral transforming sequence b
36	0.0000977	0.148	1.682	204466_s_at	SNCA	synuclein, alpha (non A4 component of amyloid precursor)
37	0.0001040	0.154	1.249	206862_at	ZNF254	zinc finger protein 254
38	0.0001140	0.161	1.384	202144_s_at	ADSL	adenylosuccinate lyase
39	0.0001146	0.161	1.548	225246_at	STIM2	stromal interaction molecule 2

Table 5 – (continued)

Rank	*Parametric P value*	*FDR*	*Fold-change*	*Probe set*	*Gene symbol*	*Description*
40	0.0001243	0.170	1.504	204964_s_at	SSPN	sarcospan (Kras oncogene-associated gene)
41	0.0001274	0.170	1.341	236796_at	BACH2	BTB and CNC homology 1, basic leucine zipper transcription factor 2
42	0.0001375	0.177	1.364	206240_s_at	ZNF136	zinc finger protein 136
43	0.0001402	0.177	2.443	202468_s_at	CTNNAL1	catenin (cadherin-associated protein), alpha-like 1
44	0.0001424	0.177	1.724	229307_at	ANKRD28	ankyrin repeat domain 28
46	0.0001660	0.196	1.665	214741_at	ZNF131	zinc finger protein 131
47	0.0001719	0.196	1.554	202909_at	EPM2AIP1	EPM2A (laforin) interacting protein 1
50	0.0001827	0.198	2.587	238852_at	PRRX1	paired related homeobox 1
51	0.0001846	0.198	1.892	224911_s_at	DCBLD2	discoidin, CUB and LCCL domain containing 2
52	0.0001909	0.199	1.930	214247_s_at	DKK3	dickkopf homolog 3 (Xenopus laevis)
53	0.0001927	0.199	2.111	202149_at	NEDD9	neural precursor cell expressed, developmentally down-regulated 9
54	0.0001996	0.200	1.236	242470_at	EID2B	EP300 interacting inhibitor of differentiation 2B
55	0.0002015	0.200	2.131	212190_at	SERPINE2	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2
58	0.0002382	0.211	1.233	225417_at	EPC1	enhancer of polycomb homolog 1 (Drosophila)
60	0.0002396	0.211	1.574	223519_at	ZAK	sterile alpha motif and leucine zipper containing kinase AZK
62	0.0002422	0.211	1.739	220145_at	MAP9	microtubule-associated protein 9
63	0.0002433	0.211	1.402	220917_s_at	WDR19	WD repeat domain 19
64	0.0002520	0.215	1.300	214078_at	PAK3	p21 (CDKN1A)-activated kinase 3
65	0.0002700	0.222	1.807	213954_at	KIAA0888	KIAA0888 protein
66	0.0002796	0.222	1.479	208993_s_at	PPIG	peptidylprolyl isomerase G (cyclophilin G)
67	0.0002799	0.222	1.433	209537_at	EXTL2	exostoses (multiple)-like 2
68	0.0002815	0.222	1.226	230212_at	SPRY1	sprouty homolog 1, antagonist of FGF signaling (Drosophila)
69	0.0002860	0.222	1.982	204359_at	FLRT2	fibronectin leucine rich transmembrane protein 2
70	0.0002868	0.222	1.370	221829_s_at	TNPO1	transportin 1
71	0.0002932	0.222	1.731	229228_at	CREB5	cAMP responsive element binding protein 5
72	0.0002936	0.222	1.606	215716_s_at	ATP2B1	ATPase, Ca++ transporting, plasma membrane 1
74	0.0003077	0.225	1.567	204422_s_at	FGF2	fibroblast growth factor 2 (basic)
76	0.0003123	0.225	1.550	202207_at	ARL4C	ADP-ribosylation factor-like 4C
77	0.0003203	0.225	1.385	225324_at	CRLS1	cardiolipin synthase 1
82	0.0003690	0.245	1.296	232064_at		Not available
84	0.0003894	0.249	1.742	219765_at	ZNF329	zinc finger protein 329
85	0.0003953	0.249	1.896	235102_x_at	GRAP	GRB2-related adaptor protein
86	0.0003955	0.249	1.451	218263_s_at	ZBED5	zinc finger, BED-type containing 5
87	0.0003998	0.249	1.661	233223_at	NEDD9	neural precursor cell expressed, developmentally down-regulated 9
88	0.0004024	0.249	1.736	212385_at	TCF4	transcription factor 4
94	0.0004467	0.249	1.372	202379_s_at	NKTR	natural killer-tumor recognition sequence
95	0.0004474	0.249	1.987	221958_s_at	GPR177	G protein-coupled receptor 177

Table 5 – (continued)

Rank	*Parametric P value*	*FDR*	*Fold-change*	*Probe set*	*Gene symbol*	*Description*
96	0.0004563	0.249	2.021	212233_at	MAP1B	microtubule-associated protein 1B
98	0.0004632	0.249	1.470	229504_at	RAB23	RAB23, member RAS oncogene family
100	0.0004788	0.249	1.377	214212_x_at	PLEKHC1	pleckstrin homology domain containing, family C (with FERM domain) member 1
101	0.0004809	0.249	1.529	212985_at		Not available
102	0.0004847	0.249	1.319	218724_s_at	TGIF2	TGFB-induced factor homeobox 2
103	0.0004848	0.249	1.681	221898_at	PDPN	podoplanin
105	0.0004878	0.249	1.358	207719_x_at	CEP170	centrosomal protein 170kDa
106	0.0004879	0.249	1.434	201363_s_at	IVNS1ABP	influenza virus NS1A binding protein
108	0.0004977	0.249	1.639	209763_at	CHRDL1	chordin-like 1
110	0.0005001	0.249	1.976	205498_at	GHR	growth hormone receptor
111	0.0005223	0.251	1.871	232113_at		Not available
115	0.0005310	0.251	1.337	215164_at	TCF4	transcription factor 4
116	0.0005320	0.251	1.783	222313_at	CNOT2	CCR4-NOT transcription complex, subunit 2
118	0.0005771	0.261	1.467	224763_at	RPL37	ribosomal protein L37
119	0.0005776	0.261	1.610	209204_at	LMO4	LIM domain only 4
120	0.0005823	0.261	1.408	227847_at	EPM2AIP1	EPM2A (laforin) interacting protein 1
121	0.0005829	0.261	1.539	208663_s_at	TTC3	tetratricopeptide repeat domain 3
122	0.0005896	0.261	1.200	230578_at	ZNF471	zinc finger protein 471
124	0.0006142	0.261	1.892	202196_s_at	DKK3	dickkopf homolog 3 (Xenopus laevis)
125	0.0006208	0.261	1.483	239768_x_at		Not available
127	0.0006305	0.261	2.216	204105_s_at	NRCAM	neuronal cell adhesion molecule
128	0.0006319	0.261	1.308	212970_at	APBB2	amyloid beta (A4) precursor protein-binding, family B, member 2
132	0.0006388	0.261	1.556	232063_x_at	FARSB	phenylalanyl-tRNA synthetase, beta subunit
133	0.0006487	0.261	2.005	220253_s_at	LRP12	low density lipoprotein-related protein 12
134	0.0006488	0.261	1.265	226843_s_at	PAPD5	PAP associated domain containing 5
135	0.0006501	0.261	1.563	211698_at	EID1	EP300 interacting inhibitor of differentiation 1
136	0.0006511	0.261	1.715	213425_at	WNT5A	wingless-type MMTV integration site family, member 5A
139	0.0006907	0.266	1.468	208661_s_at	TTC3	tetratricopeptide repeat domain 3
140	0.0006972	0.266	1.686	229530_at	GUCY1A3	guanylate cyclase 1, soluble, alpha 3
142	0.0006992	0.266	1.645	219174_at	IFT74	intraflagellar transport 74 homolog (Chlamydomonas)
143	0.0007020	0.266	2.073	209289_at	NFIB	nuclear factor I/B
144	0.0007035	0.266	1.166	210742_at	CDC14A	CDC14 cell division cycle 14 homolog A (S. cerevisiae)
145	0.0007101	0.266	1.438	209737_at	MAGI2	membrane associated guanylate kinase, WW and PDZ domain containing 2
146	0.0007116	0.266	1.931	204463_s_at	EDNRA	endothelin receptor type A
150	0.0007383	0.266	1.262	200702_s_at	DDX24	DEAD (Asp-Glu-Ala-Asp) box polypeptide 24
151	0.0007394	0.266	1.523	223463_at	RAB23	RAB23, member RAS oncogene family
152	0.0007406	0.266	1.300	225565_at	FAM119A	family with sequence similarity 119, member A

Table 5 – (continued)

Rank	*Parametric P value*	*FDR*	*Fold-change*	*Probe set*	*Gene symbol*	*Description*
154	0.0007903	0.280	1.611	218788_s_at	SMYD3	SET and MYND domain containing 3
155	0.0007943	0.280	1.161	241002_at		Not available
156	0.0008043	0.282	1.567	235368_at	ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5 (aggrecanase-2)
157	0.0008090	0.282	4.220	211756_at	PTHLH	parathyroid hormone-like hormone
158	0.0008157	0.282	1.288	212746_s_at	CEP170	centrosomal protein 170kDa
159	0.0008251	0.282	3.270	226847_at	FST	follistatin
161	0.0008325	0.282	1.610	205609_at	ANGPT1	angiopoietin 1
163	0.0008462	0.282	1.559	201810_s_at	SH3BP5	SH3-domain binding protein 5 (BTK-associated)
164	0.0008593	0.282	1.412	1556543_at	ZCCHC7	zinc finger, CCHC domain containing 7
167	0.0008649	0.282	1.501	230424_at	C5orf13	chromosome 5 open reading frame 13
168	0.0008661	0.282	1.278	210438_x_at	TROVE2	TROVE domain family, member 2
169	0.0008764	0.284	1.680	205381_at	LRRC17	leucine rich repeat containing 17
170	0.0009009	0.284	2.125	209290_s_at	NFIB	nuclear factor I/B
171	0.0009055	0.284	1.745	234996_at	CALCRL	calcitonin receptor-like
173	0.0009087	0.284	2.649	230493_at	TMEM46	transmembrane protein 46
174	0.0009110	0.284	3.152	231867_at	ODZ2	odz, odd Oz/ten-m homolog 2 (Drosophila)
175	0.0009192	0.284	1.377	225735_at	ANKRD50	ankyrin repeat domain 50
176	0.0009212	0.284	1.305	219078_at	GPATCH2	G patch domain containing 2
178	0.0009236	0.284	1.507	224989_at		Not available
179	0.0009406	0.287	1.466	202150_s_at	NEDD9	neural precursor cell expressed, developmentally down-regulated 9
180	0.0009579	0.288	1.668	202133_at	WWTR1	WW domain containing transcription regulator 1
181	0.0009606	0.288	1.435	208670_s_at	EID1	EP300 interacting inhibitor of differentiation 1
182	0.0009666	0.288	1.911	204686_at	IRS1	insulin receptor substrate 1
183	0.0009670	0.288	1.434	202132_at	WWTR1	WW domain containing transcription regulator 1
184	0.0009679	0.288	1.416	225961_at	KLHDC5	kelch domain containing 5
186	0.0009892	0.289	1.329	243305_at		Not available
188	0.0009983	0.289	1.447	242300_at	UBB	ubiquitin B

Upregulated in N+

Rank	*Parametric P value*	*FDR*	*Fold-change*	*Probe set*	*Gene symbol*	*Description*
2	0.0000021	0.042	0.346	220013_at	ABHD9	abhydrolase domain containing 9
6	0.0000085	0.077	0.635	223540_at	PVRL4	poliovirus receptor-related 4
14	0.0000330	0.125	0.766	239377_at	MGC11102	hypothetical protein MGC11102
27	0.0000703	0.141	0.760	204188_s_at	RARG	retinoic acid receptor, gamma

Table 5 – (continued)

Rank	*Parametric P value*	*FDR*	*Fold-change*	*Probe set*	*Gene symbol*	*Description*
33	0.0000876	0.145	0.767	208104_s_at	TSC22D4	TSC22 domain family, member 4
35	0.0000959	0.148	0.738	239825_at	ATF6	activating transcription factor 6
45	0.0001493	0.181	0.785	212147_at	SMG5	Smg-5 homolog, nonsense mediated mRNA decay factor (C. elegans)
48	0.0001721	0.196	0.749	218928_s_at	SLC37A1	solute carrier family 37 (glycerol-3-phosphate transporter), member 1
49	0.0001775	0.198	0.646	205204_at	NMB	neuromedin B
56	0.0002063	0.201	0.620	238804_at		Not available
57	0.0002209	0.211	0.702	209679_s_at	LOC57228	small trans-membrane and glycosylated protein
59	0.0002395	0.211	0.760	210678_s_at	AGPAT2	1-acylglycerol-3-phosphate O-acyltransferase 2 (lysophosphatidic acid acyltransferase, beta)
61	0.0002421	0.211	0.837	215106_at	TTC22	tetratricopeptide repeat domain 22
73	0.0002965	0.222	0.821	235234_at	FLJ36874	FLJ36874 protein
75	0.0003083	0.225	0.205	213240_s_at	KRT4	keratin 4
78	0.0003206	0.225	0.755	237063_at		Not available
79	0.0003300	0.228	0.847	220335_x_at	CES3	carboxylesterase 3 (brain)
80	0.0003346	0.229	0.805	239230_at	HES5	hairy and enhancer of split 5 (Drosophila)
81	0.0003464	0.234	0.636	209261_s_at	NR2F6	nuclear receptor subfamily 2, group F, member 6
83	0.0003720	0.245	0.615	1557944_s_at	CTNND1	catenin (cadherin-associated protein), delta 1
89	0.0004172	0.249	0.707	229493_at	HOXD9	homeobox D9
90	0.0004215	0.249	0.851	236676_at	NUDCD3	NudC domain containing 3
91	0.0004255	0.249	0.756	206949_s_at	RUSC1	RUN and SH3 domain containing 1
92	0.0004286	0.249	0.648	235871_at	LIPH	lipase, member H
93	0.0004387	0.249	0.666	205977_s_at	EPHA1	EPH receptor A1
97	0.0004607	0.249	0.757	1555784_s_at	IRAK1	interleukin-1 receptor-associated kinase 1
99	0.0004724	0.249	0.744	220599_s_at	CARD14	caspase recruitment domain family, member 14
104	0.0004856	0.249	0.838	207566_at	MR1	major histocompatibility complex, class I-related
107	0.0004928	0.249	0.857	1563147_at		Not available
109	0.0004986	0.249	0.662	211240_x_at	CTNND1	catenin (cadherin-associated protein), delta 1
112	0.0005283	0.251	0.784	231788_at	GPR92	G protein-coupled receptor 92
113	0.0005286	0.251	0.790	236725_at	WWC1	WW and C2 domain containing 1
114	0.0005291	0.251	0.799	232608_x_at	CARD14	caspase recruitment domain family, member 14
117	0.0005408	0.253	0.554	1553611_s_at	FLJ33790	hypothetical protein FLJ33790
123	0.0006007	0.261	0.828	218749_s_at	SLC24A6	solute carrier family 24 (sodium/potassium/calcium exchanger), member 6
126	0.0006225	0.261	0.422	206595_at	CST6	cystatin E/M
129	0.0006343	0.261	0.778	1553072_at	BNIPL	BCL2/adenovirus E1B 19kD interacting protein like
130	0.0006354	0.261	0.678	222809_x_at	C14orf65	chromosome 14 open reading frame 65
131	0.0006384	0.261	0.712	207525_s_at	GIPC1	GIPC PDZ domain containing family, member 1
137	0.0006534	0.261	0.828	231248_at	CST6	cystatin E/M
138	0.0006787	0.266	0.655	220289_s_at	AIM1L	absent in melanoma 1-like

Table 5 – (continued)

Rank	*Parametric P value*	*FDR*	*Fold-change*	*Probe set*	*Gene symbol*	*Description*
141	0.0006973	0.266	0.813	1487_at	ESRRA	estrogen-related receptor alpha
147	0.0007208	0.266	0.701	203918_at	PCDH1	protocadherin 1
148	0.0007290	0.266	0.776	204827_s_at	CCNF	cyclin F
149	0.0007310	0.266	0.626	216010_x_at	FUT3	fucosyltransferase 3 (galactoside 3(4)-L-fucosyltransferase, Lewis blood group)
153	0.0007781	0.278	0.845	220962_s_at	PADI1	peptidyl arginine deiminase, type I
160	0.0008325	0.282	0.678	230252_at	GPR92	G protein-coupled receptor 92
162	0.0008440	0.282	0.748	236616_at		Not available
165	0.0008616	0.282	0.695	235988_at	GPR110	G protein-coupled receptor 110
166	0.0008645	0.282	0.645	1552685_a_at	GRHL1	grainyhead-like 1 (Drosophila)
172	0.0009064	0.284	0.280	203757_s_at	CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)
177	0.0009227	0.284	0.724	235095_at	CCDC64B	coiled-coil domain containing 64B
185	0.0009826	0.289	0.873	233154_at	AFF3	AF4/FMR2 family, member 3
187	0.0009963	0.289	0.696	226638_at	ARHGAP23	Rho GTPase activating protein 23

The prognostic value of TRAIL and its death receptors in cervical cancer

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Abstract

Purpose: Preclinical data indicate a synergistic effect on apoptosis between irradiation and recombinant human (rh) tumor necrosis factor-related apoptosis inducing ligand (TRAIL), making the TRAIL death receptors (DR) interesting drug targets. The aim of our study was to analyze the expression of DR4, DR5, and TRAIL in cervical cancer and to determine their predictive and prognostic value.

Methods and materials: Tissue microarrays were constructed from tumors of 645 cervical cancer patients treated with surgery and/or (chemo)radiation between 1980 and 2004. DR4, DR5, and TRAIL expression in the tumor was studied by immunohistochemistry and correlated to clinicopathological variables, response to radiotherapy, and disease-specific survival.

Results: Cytoplasmatic DR4, DR5, and TRAIL immunostaining were observed in cervical tumors from 99%, 88%, and 81% of the patients, respectively. In patients treated primarily with radiotherapy, TRAIL-positive tumors less frequently obtained a pathological complete response than TRAIL-negative tumors (66.3% vs. 79.0%; in multivariate analysis: odds ratio=2.09; $P \leq 0.05$). DR4, DR5, and TRAIL expression were not prognostic for disease-specific survival.

Conclusions: Immunostaining for DR4, DR5, and TRAIL is frequently observed in the cytoplasm of tumor cells in cervical cancer patients. Absence of TRAIL expression was associated with a higher pathological complete response rate to radiotherapy. DR4, DR5, or TRAIL were not prognostic for disease-specific survival.

Introduction

Cervical cancer is a major health problem, especially in non-industrialized countries. Although prevention and early detection are the most important factors in the fight against cervical cancer, improvement of current treatment is still needed. The choice of treatment depends on the stage of the tumor. For the smaller tumors confined to the cervix (stage Ia2 and Ib1), the treatment of choice is surgery or radiotherapy with excellent 5 years survival rates (85%-95%) (1). Treatment of locally advanced cervical cancer (stage Ib2-IVa) consists of radiotherapy in combination with cisplatin-based chemotherapy. Despite improvement in survival of cervical cancer, patients treated with chemoradiation the 5-year overall survival is still approximately 52%, and treatment may be accompanied by substantial morbidity (2,3). Further improvement in survival by intensification of the standard treatment is limited by intrinsic and acquired tumor resistance to radiotherapy and/or chemotherapy and may increase short- and long-term side effects. Therefore, new alternatives are needed that can improve the antitumor effect with acceptable or no increase of toxic side effects. Tumor resistance to (chemo)radiation is commonly caused by a loss of the ability of tumor cells to go into apoptosis. Modulation of specific molecular pathways leading to increased cell death could potentially widen the therapeutic window (4). The extrinsic apoptotic pathway is initiated by activation of death receptors (DRs) expressed on the cell membrane. Several human DRs have been identified that belong to the tumor necrosis factor (TNF) receptor super family (5,6). Apoptosis is triggered by the binding of specific TNF super family ligands, such as tumor necrosis factor-related apoptosis inducing ligand (TRAIL), to their cognate receptors DR4 and DR5, respectively. DR activation results in the formation of an intracellular death-inducing signaling complex composed of trimerized receptor molecules, recruited Fas-associated death domain molecules and procaspase 8 molecules (5,6). After assembly of the death-inducing signaling complex, a caspase-8 initiated intracellular apoptotic cascade is activated, leading to cleavage of several substrates in the cytoplasm and nucleus and completion of the apoptotic program (5,6).

Preclinical work from our own group indicates that the combination of targeting the TRAIL pathway by exposure to agonistic DR4 and DR5 antibodies or recombinant human (rh)TRAIL and irradiation works synergistically in cervical cancer cells (7). Moreover, in early clinical trials, these drugs also can be safely administered even when combined with chemotherapy (8,9). We previously reported in a small series (25 patients) that cervical tumors frequently stain positive for DR4, DR5, and TRAIL (10). However, their role in relation to clinical outcome in cervical cancer is still unknown. Previous work by our group showed that DR4, DR5, and TRAIL staining in normal

cervical tissue was associated with undifferentiated cells in the basal and parabasal layer, whereas in cervical cancer, the staining was more homogeneous (10). TRAIL expression has been found in a wide range of normal tissues but is able to induce apoptosis only in transformed and malignant cells (11,12). Therefore, we concentrated on tumor specimens in this study.

We analyzed, the presence of DR4, DR5, and TRAIL in tumors of a large cohort of cervical cancer patients with stage Ia2-IVa disease treated with surgery and or (chemo)radiation. We combined classical clinical histopathological characteristics and immunostaining for TRAIL and its pro-apoptotic receptors to identify possible relations to each other and to treatment outcome. We also investigated DR4, DR5, and TRAIL expression in relation to response for patients treated with radiotherapy with or without chemotherapy.

Materials and methods

Patients

Clinicopathological characteristics of all cervical cancer patients referred to the Department of Gynecological Oncology of the University Medical Center Groningen are prospectively stored in a database since 1980. All staging and surgical procedures were performed at the University Medical Center Groningen. For the present cohort study, all patients diagnosed with nonmetastatic invasive cervical cancer who were treated between January 1980 and December 2004 were identified. Eligibility was based on diagnosis of invasive cervical cancer stage Ia2-IVa treated with a Wertheim-Meigs operation or with radiotherapy plus or minus chemotherapy. We considered surgery as primary treatment in those patients in whom a Wertheim-Meigs operation was performed whether or not this was followed by radiation or chemoradiation. Primary radiotherapy was defined as radiotherapy or chemoradiation as the first treatment modality despite an additive surgical procedure. The clinicopathological and follow-up data were obtained during standard treatment and follow-up of the patients. For the present study, all relevant data were retrieved from our database into a separate anonymous database. The identity of the patients was protected by study-specific, patient codes. In case of uncertainties with respect to clinicopathological and follow-up data, the larger database could only be checked through the department's data managers. Follow-up data was collected up to November 2007.

Staging and treatment

Bimanual examination under general anesthesia was performed for clinical staging, in accordance with the Fédération Internationale de Gynécologie Obstétrique (FIGO) guidelines. Patients were treated according to the time period prevailing protocol, mainly based on FIGO staging. In general, this was a Wertheim-Meigs operation for stage Ib/IIa cervical cancer patients followed by external beam radiotherapy (EBRT) up to 45 Gray (Gy) in case of lymph node metastases, parametrial invasion, or positive resection margins. For the higher stage patients, primary treatment was either radiation (EBRT up to 45 Gy and low-dose-rate brachytherapy, two applications of 17.5 Gy) or chemoradiation.

EBRT was delivered by a linear accelerator. A box technique was used comprising an anterior, a posterior, and two lateral fields. The superior field border was the upper border of the fourth lumbar vertebra; the lowest field border was the lower margin of the obturator foramen (or in stage IIIa, the distal vagina). The lateral margin of the anterior-posterior field was 2 cm lateral from the transverse diameter of the pelvic brim. The ventral border of the lateral fields is the upper margin of the symphysis and the dorsal margin the front of the os coccyx. All fields were given daily, 5 days a week. The dose was 1.8 Gy given to the center and planned on a contour outline of the patient. Low dose rate (^{137}Cesium) brachytherapy was applied with a standard applicator with a dose of 17.5 Gy to point A (reference location 2 cm lateral and 2 cm superior to the cervical os). If brachytherapy was impossible or inappropriate in cases of tumor extension into the parametria or lymph nodes, patients received an additional external boost of 25.2 Gy to a total dose of 70.2 Gy.

Concurrent chemotherapy before 1999 consisted of three 4-weekly cycles of carboplatin 300 mg/m² Day 1 and fluorouracil (5-FU) 600 mg/m² Days 2-5 intravenously (IV). After 1999, chemotherapy consisted of cisplatin 40 mg/m² IV once weekly for 6 weeks concomitant with external pelvic and intracavitary radiation.

In the period up to 1993, following irradiation all patients, if technically operable and in the absence of extra-uterine disease, underwent an additive hysterectomy, whereas in the period thereafter, only comparable patients with residual tumor identified by routine biopsy 6-10 weeks after completion of irradiation were operated.

Pathological response evaluation after primary radiotherapy

Pathological response to primary radiotherapy was evaluated in the hysterectomy material in the time period prior to 1994 and, in the period thereafter, through biopsy

6-10 weeks after completion of irradiation. Pathological complete response was defined as absence of tumor cells in postirradiation tissue. Chemotherapy was included in the response analysis as an independent factor.

Tissue microarray (TMA)

From the patients meeting the inclusion criteria, we collected the paraffin-embedded tumor material and the hematoxylin-eosin (H&E)-stained slides obtained at diagnostic procedure or the specimens from patients at primary surgery. On the H&E-stained slides, representative tumor areas were marked avoiding areas of necrosis or severe leukocyte infiltration. From the corresponding paraffin blocks, three cores of 0.6 mm diameter were taken and placed in predefined array locations in a recipient blank paraffin block, using a precision instrument (Beecher Instruments, Silver Spring, MD). Eleven arrays were constructed, each containing three cores per tumor. Each array also contained internal control tissue such as normal cervix, skin epithelium, colon polyps, breast cancer, colon cancer, ovarian cancer, and several cervical cancer specimens.

Immunohistochemistry

Staining procedures for DR4, DR5, and TRAIL were performed as described previously (10,13). Briefly, sections (4 μm) were deparaffinized in xylene and endogenous peroxidase was blocked by incubation in 0.3% hydrogen peroxide for 30 min. Only DR5 needed antigen retrieval by 15-min microwave treatment in 10 mM citric acid, pH 6.0 at 95-100ºC. All primary antibodies were diluted in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 1% AB serum and added to the deparaffinized tumor material for 1 h at room temperature. Primary antibodies and dilutions were for DR4 goat anti-DR4 polyclonal antibody (1:50, clone C-20, Santa Cruz Biotechnology, Santa Cruz, CA), for DR5 rabbit anti-DR5 polyclonal antibody (1:100, Oncogene Research, Cambridge, MA) and for TRAIL goat anti-TRAIL polyclonal antibody (1:25, clone K18, Santa Cruz Biotechnology). 3,30-Diaminobenzidine (DAB) was used as chromogen, and the slides were counterstained in hematoxylin. For the negative controls, the primary antibody was replaced by normal goat IgG (DR4 and TRAIL) or normal rabbit IgG (DR5).

Analysis of immunohistochemical staining

For DR4, DR5, and TRAIL staining, intensity was semiquantitatively scored: no staining (0), weakly positive (1), positive (2), or intense (3). For statistical analysis, all cases

were initially studied separately and then dichotomized. Samples with scores 0 and 1 were regarded as negative and samples with score 2 and 3 as positive. Because of the relatively high amount of patients with no staining for TRAIL, we also divided the group in negative (0) and positive (1-3). (When referring to this classification this will be clearly stated in the results.) The cellular localization (nuclear, membranous, or cytoplasmatic) and patterns of staining (homogenous or heterogeneous) were also recorded. Two independent observers (KAH, JHM) scored the TMAs, and a concordance of more than 95% was found. The discordant cases were reviewed, and scores were reassigned on consensus of opinion. Patients were only included in the analysis in cases of minimally two representative tissue cores on the TMA.

Statistical analysis

Statistical analysis was performed with SPSS 14.0 for Windows (SPSS, Chicago, IL). A *P* value of $\leq$0.05 was considered statistically significant. The population was analyzed as a whole, and the group treated primarily with radiotherapy was analyzed separately. Associations between positive protein expression (DR4, DR5, and TRAIL, respectively; as dependent factor) and clinicopathological characteristics (age, stage, histology, tumor differentiation, lymphovascular invasion, and tumor volume; as independent factors) were calculated using logistic regression analyses. For the clinicopathologic analysis, the following covariates were used: age (continuous variable), FIGO stage ($\geq$IIb vs. <IIb), histology (adenocarcinoma vs. squamous cell carcinoma), tumor differentiation (poor vs good and moderate), lymphovascular invasion (present vs. absent), and tumor volume ($\geq$4 cm vs. <4 cm). Variables that were significant ($P\leq$0.1) in univariate analysis were included in multivariate analysis in a stepwise manner. To determine factors predicting for response to radiotherapy, presence of pathological complete response to radiotherapy (as dependent factor) was evaluated in relation to clinicopathologic factors, protein staining, and the use of chemotherapy (as independent factors) with logistic regression analysis. Disease-specific survival (DSS) was defined as time period from date of diagnosis up to time point of death due to cervical cancer or last documented contact being alive. DSS was calculated for the whole population as well as for all patients in the primary radiotherapy subgroup. Differences in DSS according to clinicopathologic characteristics and to expression of DR4, DR5, and TRAIL were analyzed using the Cox regression analysis. Variables with a *P* value $\leq$0.1 in the univariate analyses were included in the multivariate analyses. Elimination of variables in a stepwise manner identified the statistically significant predictors on DSS by using a multivariate analysis.

Table 1 – Patient characteristics

Variables	Surgery		(Chemo*)RT†		Total	
	n	%	*n*	%	*n*	%
Patients	313	49%	332	51%	645	100%
Age at diagnosis (years)						
Median	43.0		54.1		47.7	
Range	17.5 – 86.2		20.6 – 92.0		17.5 – 92.0	
Follow-up (months)						
Median	64.9		45.9		55.7	
Range	3.7 – 223.2		1.5 – 219.7		1.5 – 223.2	
FIGO stage						
Ia2	1	0%	0	0%	1	0%
Ib1	198	63%	38	11%	236	37%
Ib2	63	20%	19	6%	82	13%
IIa	50	16%	46	14%	96	15%
IIb	1	0%	161	48%	162	25%
IIIa	0	0%	7	2%	7	1%
IIIb	0	0%	47	14%	47	7%
IVa	0	0%	14	4%	14	2%
Histology						
Squamous	220	70%	282	85%	502	78%
Adenocarcinoma	85	27%	46	14%	131	20%
Other	8	3%	4	1%	12	2%
Treatment						
Post operative RT†	103	34%				
Post operative chemo* RT†	18	6%				
Chemo*			151	45%		

*Chemotherapy
†Radiotherapy

Results

Patient's characteristics

In total, we identified 765 patients, in 645 of whom sufficient tumor material was available to construct the TMA.

Clinicopathological data of the 645 patients with sufficient tumor material is summarized in Table 1. The median follow-up time was 55.7 months, and the median age at diagnosis 51.3 years. Primary treatment consisted of surgery in 313 patients (49%) and radiotherapy in 332 patients (51%). The baseline characteristics of the 120 patients in whom tumor material was missing differed from the study population re-

garding treatment modality primary radiotherapy (P<0.001) and FIGO staging (higher stage; P<0.001). This imbalance is accounted for by the fact that there was more tissue available from operated patients with lower tumor stage than from patients with only a biopsy taken before start of radiotherapy.

Immunohistochemical staining for DR4, DR5, and TRAIL

The results of the immunostainings are shown in Table 2. The number of patients with less than two representative tissue cores was 5.3% for DR4, 5.1% for DR5, and 4.7% for TRAIL. All three protein stainings were cytoplasmatic with no apparent membranous staining (Fig. 1). DR4 and DR5 were at least weak positive in 87.9% and 98.9% of the cases; for TRAIL, this was found in 79.8% of the cases. The expression of all the three proteins correlated with each other apart from TRAIL (negative [score 0] vs. positive [score 1-3]) and DR5 (negative [score 0-1] vs. positive [score 1-3]).

DR4, DR5, and TRAIL protein staining in relation to clinicopathologic characteristics

Table 3 shows the odds ratio (OR)s for the different immunostaining parameters in relation to known clinicopathologic characteristics in a uni- and multivariate logistic regression analysis. DR4 positive staining was associated with low tumor stage (<IIb) and presence of adenocarcinoma. DR5 positive staining was related to high tumor stage (≥IIb) and to the presence of adenocarcinoma. No association was found between TRAIL expression and clinicopathologic characteristics.

Table 2 – Staining distribution

Tumor expression	DR*4		DR*5		TRAIL†	
	n	%	*n*	%	*n*	%
Negative	74	11%	7	1%	124	19%
Weak positive	166	26%	158	25%	223	34%
Positive	358	56%	439	68%	264	41%
Strong positive	13	2%	8	1%	4	1%
Missing cores	34	5%	33	5%	30	5%
Total	**645**		**645**		**645**	

*Death receptor
†Tumor necrosis factor-Related Apoptosis Inducing Ligand

Figure 1 – Immunohistochemical staining for DR4, DR5, and TRAIL in a tissue microarray with tumors from cervical cancer patients.

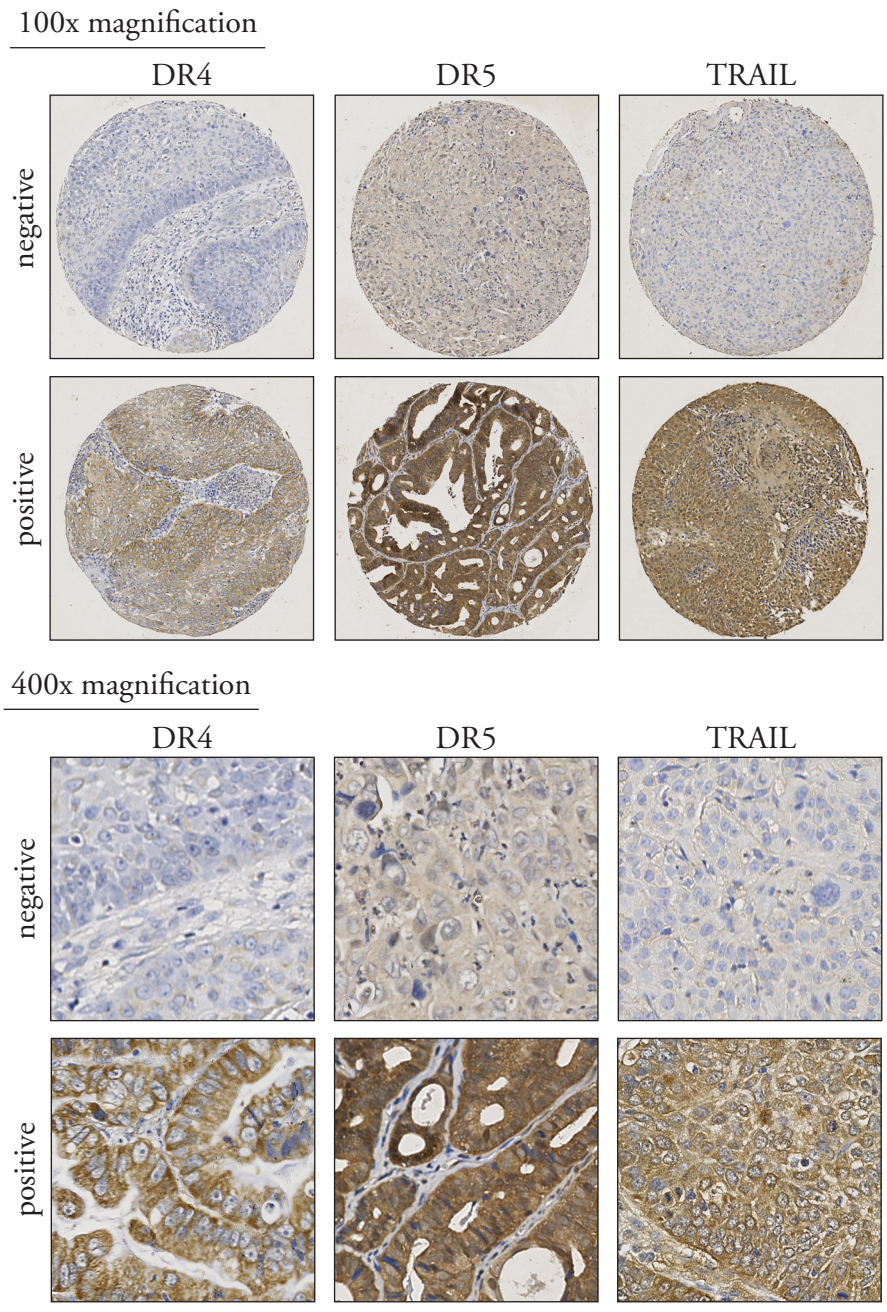

Table 3 – Association between DR4 (A), DR5 (B) and TRAIL (C) staining in relation to clinicopathologic characteristics

A) DR4 positive n=645	**Univariate** *OR* (95%CI)†*	**Multivariate** *OR* (95%CI)†*
Age	**0.98 (0.97 – 0.99)‡**	**0.99 (0.98 – 1.00)§**
Stage ≥IIb	**0.52 (0.37 – 0.73)‡**	**0.60 (0.41 – 0.84)§**
Adenocarcinoma	**2.76 (1.74 – 4.39)‡**	**2.48 (1.55 – 3.97)§**
Poor differentiation	0.76 (0.45 – 1.29)	¶
Lymphovascular invasion	1.34 (0.93 – 1.92)	¶
Tumor volume ≥4 cm	**0.59 (0.42 – 0.82)‡**	¶
B) DR5 positive n=645	**Univariate** *OR* (95%CI)†*	**Multivariate** *OR* (95%CI)†*
Age	**1.01 (1.00 – 1.02)‡**	¶
Stage ≥IIb	**3.23 (2.09 – 4.97)‡**	**3.61 (2.16 – 6.02)§**
Adenocarcinoma	**1.73 (1.05 – 2.84)‡**	**1.90 (1.13 – 3.18)§**
Poor differentiation	0.92 (0.64 – 1.34)	¶
Lymphovascular invasion	**0.51 (0.35 – 0.75)‡**	0.68 (0.45 – 1.02)
Tumor volume ≥4 cm	**1.43 (0.99 – 1.43)‡**	¶
C) TRAIL positive n=645	**Univariate** *OR* (95%CI)†*	**Multivariate** *OR* (95%CI)†*
Age	1.00 (0.99 – 1.01)	¶
Stage ≥IIb	1.25 (0.90 – 1.74)	¶
Adenocarcinoma	0.91 (0.61 – 1.36)	¶
Poor differentiation	0.84 (0.60 – 1.18)	¶
Lymphovascular invasion	0.96 (0.68 – 1.35)	¶
Tumor volume ≥4 cm	1.05 (0.76 – 1.46)	¶

Staining for DR4, DR5 and TRAIL with scores 0 and 1 were regarded to be negative and samples with score 2 and 3 were regarded to be positive for DR4, DR5 and TRAIL

*Odds Ratio
†95% confidence interval
‡*P* value <0.1
§*P* value <0.05
¶Not included in multivariate analysis

Table 4 – Response to radiotherapy or chemoradiation. Correlation between absence of complete response and clinicopathologic factors.

n=243	Univariate		Multivariate	
	*OR**	*(95%CI)*†	*OR**	*(95%CI)*†
Age	0.99	(0.97 – 1.01)	¶	
Stage ≥IIb	**1.75**	**(0.95 – 3.25)**‡	**2.03**	**(1.06 – 3.89)**§
Chemotherapy	0.77	(0.44 – 1.34)	¶	
Adenocarcinoma	**2.28**	**(1.10 – 4.75)**‡	**2.22**	**(1.05 – 4.70)**§
Poor differentiation	0.69	(0.29 – 1.18)	¶	
Lymphovascular invasion	1.06	(0.49 – 2.26)	¶	
Tumor volume ≥4 cm	1.44	(0.75 – 2.78)	¶	
DR4 expression positive	1.13	(0.65 – 1.98)	¶	
DR5 expression positive	1.78	(0.73 – 4.30)	¶	
TRAIL expression positive	1.13	(0.65 – 1.97)	¶	
TRAIL expression• positive	**1.91**	**(0.94 – 3.87)**‡	**2.09**	**(1.01 – 4.33)**§

*Odds Ratio
†95% confidence interval
‡*P* value <0.1
§*P* value <0.05
¶Not included in multivariate analysis
•Score 0 (negative) vs. 1-3 (positive)

Pathologic complete response to radiotherapy in relation to DR4, DR5, and TRAIL protein staining

To relate expression of DR4, DR5, and TRAIL and clinicopathologic characteristics to pathologic complete response to radiotherapy a uni- and multivariate logistic regression analysis was performed on 243 patients in whom either a routine hysterectomy was performed (until 1996) or in whom routine posttreatment biopsies were taken (after 1996; Table 4). The patient group eligible for posttreatment response evaluation was younger (median, 50.4 years) than the group with no posttreatment evaluation (median, 67.8 years; $P<0.001$). For all other clinicopathologic characteristics, there was no difference between the patients with or without posttreatment evaluation, suggesting patients operability as the most important reason for not performing a posttreatment biopsy. Patients not fit for additive surgery were not evaluated with a posttreatment biopsy. In multivariate analysis, the absence of a pathological complete response in this group of 243 patients was related to positive staining for TRAIL (score 1-3), presence

Table 5 – Disease-specific survival

A) Whole population n=645	Univariate HR* (95%CI)†		Multivariate HR* (95%CI)†	
Age	**1.02**	**(1.01 – 1.02)**‡	**1.01**	**(1.00 – 1.02)**§
Stage ≥IIb	**3.20**	**(2.41 – 4.25)**‡	**2.30**	**(1.65 – 3.20)**§
Adenocarcinoma	1.13	(0.80 – 1.59)	¶	
Poor differentiation	1.26	(0.94 – 1.67)	¶	
Lymphovascular invasion	0.96	(0.70 – 1.31)	¶	
Tumor volume ≥4 cm	**3.01**	**(2.20 – 4.12)**‡	**2.01**	**(1.41 – 2.86)**§
DR4 expression positive	0.93	(0.69 – 1.23)	¶	
DR5 expression positive	**1.69**	**(1.18 – 2.41)**‡	¶	
TRAIL expression positive	1.11	(0.83 – 1.47)	¶	
TRAIL expression• positive	0.98	(0.69 – 1.38)	¶	
B) Primary radiotherapy group n=332	**Univariate** HR* (95%CI)†		**Multivariate** HR* (95%CI)†	
Age	1.00	(0.99 – 1.01)	¶	
Stage ≥IIb	**2.46**	**(1.60 – 3.79)**‡	**1.82**	**(1.14 – 2.89)**§
Adenocarcinoma	1.43	(0.93 – 2.20)	¶	
Poor differentiation	1.21	(0.70 – 2.08)	¶	
Lymphovascular invasion	1.14	(0.73 – 1.78)	¶	
Tumor volume ≥4 cm	**2.16**	**(1.40 – 3.34)**‡	**1.66**	**(1.05 – 2.62)**§
DR4 expression positive	1.13	(0.81 – 1.57)	¶	
DR5 expression positive	1.21	(0.75 – 1.94)	¶	
TRAIL expression positive	0.99	(0.71 – 1.38)	¶	
TRAIL expression• positive	1.15	(0.77 – 1.71)	¶	
Residual tumor	**5.05**	**(3.62 – 7.04)**‡	**4.50**	**(3.17 – 6.38)**§
Chemotherapy	**0.70**	**(0.50 – 0.97)**‡	**0.62**	**(0.43 – 0.87)**§

*Hazard Ratio
†95% confidence interval
‡*P* value <0.1
§*P* value <0.05
¶Not included in multivariate analysis
•Score 0 (negative) vs. 1-3 (positive)

of adenocarcinoma and patients with FIGO stage ≥IIb, ORs were, respectively, 2.09, 2.22, and 2.03 (all *P* values ≤0.05). Pathologic complete response rates were 66.3% vs. 79% for TRAIL positive vs. negative tumors, 54.3% vs. 73% for adenocarcinomas vs. squamous cell carcinomas, and 66.3% vs. 77.5% for FIGO stage ≥IIb vs. FIGO stage <IIb. Pathologic complete response to radiotherapy was not related to the concomitant use of chemotherapy. Results are shown in Table 4.

Disease-specific survival in relation to DR4, DR5, and TRAIL expression

In univariate Cox regression analysis, DR5 positive staining was associated with a worse DSS. In multivariate analysis for the whole group, age, high tumor stage, and tumor volume ≥4 cm correlated with a worse outcome. None of the immunostaining parameters (DR4, DR5, or TRAIL) correlated with disease outcome in multivariate analysis. The hazard rates (HRs) and confidence intervals (CIs) are reported in Table 5A. In the analyses of the primarily operated patients, the presence of positive pelvic lymph nodes (HR=4.99; 95%CI=2.84 – 8.76) and tumor volume (HR=1.83; 95%CI=1.07 – 3.13) were highly significant unfavorable prognostic factors.

In the primary radiotherapy group (n=332), a worse DSS was related to high tumor stage (HR=1.82; 95%CI=1.14 – 2.89), larger tumor volume (HR=1.66; 95%CI=1.05 – 2.62), and persisting tumor after treatment (HR=4.50; 95%CI=3.17 – 6.38). Although no effect on pathologic complete response chemotherapy contributed to a better DSS (HR=0.62; 95%CI=0.43 – 0.87) in multivariate analysis (Table 5B).

Discussion

This study shows that DR4, DR5, and TRAIL were cytoplasmatically expressed in most stage Ia2-IVa cervical tumors. Patients with a TRAIL-positive tumor had an absolute 12.6% lower chance to obtain a complete pathologic response after radiotherapy. Absence of a complete pathologic response to radiotherapy was also related to presence of adenocarcinoma and higher FIGO stage. In multivariate analysis, DR4, DR5, and TRAIL immunostaining were not associated with DSS.

In the population studied, we found that classic clinicopathologic characteristics such as tumor stage (≥IIb) and tumor size (≥4 cm) were the most important parameters affecting prognosis. Lymph node involvement was not taken into account in the analysis of the whole population because in most of the primarily irradiated patients, the presence of lymph node involvement is not known. In the analyses of the primarily operated patients, positive lymph nodes were associated with an unfavorable prognosis. These findings indicate the presence of a representative study population, and the distribution of patient characteristics (see Table 1) also mimics normal distribution in comparable cervical cancer populations from the Western world.

In our study, cytoplasmatic DR4, DR5, and TRAIL immunostaining were frequently observed, which was also the case in two much smaller studies not addressing the relation with DSS (10,14). The biologic meaning of cytoplasmatic DR4, DR5, and

TRAIL is not known, while for their activity they should be present at the cell membrane. Moreover, cytoplasmatic DR4 and DR5 do not exclude the presence of the DRs on the cell surface. Studies in ovarian, colon, and lung cancers have also demonstrated cytoplasmatic staining for DR4, DR5, and TRAIL (15-17). DR4, DR5, and TRAIL expression has been associated, in various tumors, with different clinical outcome. In patients with stage III colon cancer treated with surgery and adjuvant chemotherapy, high cytoplasmatic DR4 expression at diagnosis was related to worse disease-specific and overall survival (17). In a large cohort of breast cancer patients (n=655) (18) and in 95 non-small cell lung cancer patients (16), high DR5 expression was associated with worse survival. In ovarian cancer, high TRAIL expression measured by real-time polymerase chain reaction was related to a better overall survival (19). In melanoma patients, DR4 and DR5 expression were not associated with clinical outcome (20). The diversity in the prognostic value of the DRs and TRAIL in various tumor types may be related to the various tumor types tested and the differences in treatment to which the patients were exposed. The lack of prognostic significance as observed in our study does not exclude functionality of the DRs. On the basis of preclinical data, it is likely that following chemo- and/or radiotherapy, the DRs are upregulated at the cell membrane. More specifically, in our preclinical cervical cancer model, we showed by flow cytometry an upregulation of DR4 and DR5 membrane expression after irradiation (7). It might well be that during irradiation cytoplasmatic DR4 and DR5 are transferred to the membrane surface, thereby presenting them as potential targets for DR4/5 targeted drugs.

Historically, response rate to radiotherapy in cervical cancer has been measured in different ways, such as clinical examination or by postirradiation biopsies or hysterectomies, jeopardizing meaningful comparison of response rates between studies. The uniqueness of this study lies in the fact that it analyzes response evaluation to radiotherapy in relation to DR4, DR5, TRAIL and classic clinicopathologic parameters. Pathologic complete response as assessed in our study gives the earliest insight in the biology of tumor in relation to irradiation. The importance of pathologic response measurement as a predictive marker for prognosis after irradiation for cervical cancer has been well established and was reviewed by Trott (21). A study in 556 cervical cancer patients showed that clinical measurement of response divided in no gross residual tumor and gross residual tumor also correlated well with clinical outcome (22). In our population, the pathologic complete response rate was 70%. The cervical cancer patients with tumors expressing TRAIL (75.7%) experienced less often a pathological complete response than those expressing no TRAIL (66.3% vs. 78.9% OR in a multivariate analysis

2.09, *P* value ≤0.05). TRAIL expression, however, did not correlate with DSS, which in part may be caused by the fact that a proportion of patients with residual tumor after radiotherapy were salvaged by surgery, which in our institution is the standard of care for patients with resectable residual disease (23).

Not much is known about differences in radioresponsiveness between adenocarcinoma and squamous cell carcinoma in cervical cancer patients. In our study, patients with adenocarcinomas compared with squamous cell carcinomas obtained a pathologic complete response to irradiation less frequently, which has also been observed in a smaller FIGO stage Ib population (24). Despite the use of a different irradiation technique than ours, Rouzier *et al.* (25) also showed a near significant difference (*P* value 0.07) in complete pathologic response to radiotherapy between squamous cell carcinomas (62%) and adenocarcinomas (38%). It has been shown with measurements of MIB-1 and PC10 antigens in cervical cancer paraffin sections that cervical adenocarcinomas have no change, whereas squamous cell carcinomas have a transient increase in cycling cell population after 9 Gy of irradiation (26). The lower growth fraction in adenocarcinomas may be one reason for the radiation resistance of these tumors. This advantage in radioresponsiveness for squamous cell carcinomas did not translate into better DSS. As mentioned earlier, patients with residual disease after (chemo)radiation may be salvaged by surgery. Nijhuis *et al.* previously showed in a similar population that 38% of the patients not having complete response after radiotherapy still achieve long-term complete remission after salvage surgery (23).

We observed that high DR4 and DR5 expression have an opposite correlation with FIGO stage. This finding suggests that depending on tumor stage, the DR route might be targeted differently. Both high DR4 and DR5 cytoplasmatic expression correlated positively with adenocarcinoma histology. The implication of this finding is not clear and needs to be elucidated.

Preclinical data indicated that the cytotoxic effect of radiotherapy is enhanced by rhTRAIL or its agonistic antibodies (7,27). Irradiation induced both DR4 and DR5 membrane expression, whereas the enhancement of the cytotoxic effect was especially but not exclusively DR4 mediated (7). Moreover, in early clinical trials, rhTRAIL and its agonistic antibodies have been safely administered, even combined with chemotherapy (9,28,29). On the basis of the finding of our preclinical data (7), targeting DR4 in combination with (chemo)radiation appears to be the most tentative treatment option for a clinical trial. The significance of DR4, DR5, and TRAIL expression as predictive factor for response should be prospectively investigated in a TRAIL route intervention study. In cervical cancer patients, in contrast to most other tumor types, it is relatively

easily to perform serial biopsies during and after treatment allowing investigation of possible changes in expression of DR4, DR5, and TRAIL.

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Expression of EGFR and activated EGFR predict poor response to (chemo)radiation and survival in cervical cancer

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Abstract

Purpose: Activation of the EGFR signaling pathway has been reported to induce resistance to (chemo)radiation in cancers, such as head & neck cancer, while EGFR targeted agents in combination with (chemo)radiation appear to improve treatment efficacy. The aim of this study was to determine the relation between proteins involved in the EGFR pathway and response to (chemo)radiation and survival in a large, well-documented series of cervical cancer patients.

Experimental design: Pre-treatment tissue samples of 375 consecutive FIGO stage Ib-IVa cervical cancer patients treated with (chemo)radiation between January 1980 and December 2006 were collected. Clinicopathologic and follow-up data were prospectively obtained during standard treatment and follow-up. Protein expression of EGFR, pEGFR, PTEN, pAKT, and pERK was assessed by immunohistochemistry on tissue microarrays.

Results: EGFR staining was present in 35.3%, pEGFR in 19.7%, PTEN in 34.1%, pAKT in 4.1%, and pERK in 29.2% of tumors. pEGFR staining was related to PTEN (P=0.001) and to pERK staining (P=0.004). EGFR staining was inversely related to PTEN (P=0.011). In multivariate analysis, membranous staining of EGFR (HR=1.84; 95%CI=1.20 – 2.82; P=0.005) and cytoplasmic staining of pEGFR (HR=1.71; 95%CI=1.11 – 2.66; P=0.016) were independent predictors of poor response to (chemo)radiation. Membranous EGFR staining also was an independent prognostic factor for poor disease-specific survival (HR=1.54; 95%CI=1.09 – 2.17; P=0.014).

Conclusions: EGFR and pEGFR immunostaining are frequently observed and independently associated with poor response to therapy and disease-specific survival in cervical cancer patients primarily treated by (chemo)radiation. Our data presents the EGFR pathway as a promising therapeutic target in already ongoing clinical trials.

Introduction

Standard treatment of locally advanced cervical cancer has changed from radiotherapy alone to concurrent platinum-based chemoradiation. Despite this change, the 5-year survival in patients with locally advanced cervical cancer is still around 52% (1). Currently, there are no (biological) markers available that accurately predict response to (chemo)radiation.

Epidermal growth factor receptor (EGFR) is involved in the ErbB signaling pathway, which is often dysregulated in cancer. Autophosphorylation of EGFR to pEGFR leads to activation of two downstream pathways: the Ras/Raf/MEK/ERK pathway and the PI3K/AKT pathway. PTEN (Phosphatase and Tensin Homolog Deleted on Chromosome Ten) acts as a tumor suppressor gene by inhibiting phosphorylation and thereby activation of AKT (2,3). Both downstream EGFR pathways have been shown to be involved in processes associated with carcinogenesis and tumor progression, such as inhibition of apoptosis, cell migration, cell growth, and angiogenesis (4), and more recently also in conferring resistance to irradiation (5,6).

EGFR and some of its downstream targets have been studied previously in cervical cancer, but conflicting results about their prognostic significance have been reported (7-12). Expression of phosphorylated AKT (pAKT) in cervical cancer appeared to be related to local recurrence, as a measure for radiation resistance (13) and Faried et al. showed that patients with pAKT negative tumors had a more favorable prognosis (14). In contrast, Lee *et al.* found an inverse correlation for pAKT with survival (15). Hypermethylation and mutations of the *PTEN* gene have been associated with poor outcome after radiotherapy (16,17). Thus, the prognostic significance of different components of the EGFR pathway in cervical cancer is equivocal, due to small, various (frequently a mix of primarily surgically and radiotherapeutically treated) patient populations and differences in immunohistochemistry. Moreover, protein expression of EGFR and its downstream targets have not been studied before in relation to response to (chemo)radiation in cervical cancer.

In head & neck squamous cell carcinoma, protein expression of EGFR appears to be related to a higher local relapse rate, indicating a poor response to radiotherapy (18,19). Moreover, a recent randomized clinical trial showed a significant prolonged progression-free survival for head & neck squamous cell cancer patients treated with radiotherapy in combination with cetuximab, a chimeric human mouse anti-EGFR monoclonal antibody, when compared to standard radiotherapy (20). The aim of the present study

was to determine protein expression of EGFR, pEGFR, PTEN, pAKT, and pERK in relation to response to (chemo)radiation and survival in a large, well-documented series of cervical cancer patients.

Patients and methods

Patients

For the present study, all patients primarily treated by radiotherapy or chemoradiation in the University Medical Center Groningen or in collaborating hospitals between January 1980 and December 2006 were selected. Patients with stage IVb disease were excluded, as their treatment was individualized. Follow-up data was collected for at least five years or up till January 2008. Staging was performed according to FIGO guidelines. Radiotherapy included external beam radiotherapy (EBRT) up to 45 Gy and low dose rate brachytherapy, two applications of 17.5 Gy. Concurrent chemotherapy before 1999 consisted of three cycles of carboplatin and fluorouracil (5-FU). Carboplatin dose was 300 mg/m², dissolved in 250 ml 5% glucose, given over 30 minutes intravenously (i.v.) on Day 1. Fluorouracil dose was 600 mg/m², dissolved in 2 liter of saline and administered i.v. continuously on Days 2 to 5. This cycle was repeated another two times every 28 days. After 1999 chemotherapy consisted of Cisplatin 40 mg/m² i.v. once a week for 6 weeks concomitant with external pelvic and intracavitary radiation. Paraffin-embedded formalin-fixed primary tumor tissue was collected from each patient. Patients were only included in the analysis if enough tumor tissue was available for tissue microarray (TMA) construction.

Institutional Review Board approval

In the University Medical Center Groningen clinicopathologic and follow-up data are prospectively obtained during standard treatment and follow-up and stored in a computerized registration database. For the present study, all relevant data were retrieved from this computerized database into a separate, anonymous database. Patient identity was protected by study-specific, unique patient numbers. Codes were only known to two dedicated data managers, who also have daily responsibility for the larger database. In case of uncertainties with respect to clinicopathologic and follow-up data, the larger databases could only be checked through the datamanagers, thereby ascertaining the protection of patients' identity. Using the registration database all tissue specimens were identified by unique patient numbers and retrieved from the archives of the Department

of Pathology. Therefore, according to Dutch law no further Institutional Review Board approval was needed (http://www.federa.org/).

Evaluation of response to (chemo)radiation

In the period up to 1993 eligible patients underwent an additive hysterectomy 6-8 weeks after completion of (chemo)radiation. After 1993 only patients with residual disease in a biopsy taken 8-10 weeks after completion of primary treatment underwent surgery (21). Hysterectomy or biopsy after (chemo)radiation was only performed when a patient (technically) was judged to be operable. As post-(chemo)radiation biopsy and / or hysterectomy to evaluate response to (chemo)radiation) were performed in only a selected group of patients, response to (chemo)radiation was evaluated retrospectively in two models. Model I: Response to (chemo)radiation was determined by locoregional disease-free survival in all patients, which was defined as the period from diagnosis to clinical locoregional progression of disease during treatment or to locoregional recurrence after treatment. If location of recurrence was unknown, patients were not included in this analysis. Model II: In order to be able to analyze two populations with supposedly the highest difference in sensitivity to (chemo)radiation, two populations with optimal and very poor response to (chemo)radiation were defined. Patients with complete disease eridication were patients with no residual disease in their post treatment biopsy/hysterectomy specimen and who did not have a locoregional recurrence in the follow-up, with a follow-up time of at least 2 years, vs. patients with clinical evidence of disease progression during treatment or clinical evidence of disease persistence at examination after completion of primary treatment.

Tissue microarray construction

For the construction of the TMA only pre-treatment biopsies were used. Areas of representative tumor tissue were marked on hematoxylin- and eosin- (H&E) stained slides of the paraffin-embedded tissue. Areas of necrosis and areas with severe leukocyte infiltration were avoided. The TMAs were constructed by using a precision instrument (Beecher Instruments, Silver Spring, Maryland). Three cores of 0.6 mm in diameter were punched from the marked area on the paraffin-embedded tissue (donor block). These cores were then placed in a blank paraffin block (recipient block), in pre-defined locations. After inserting all the cores, the recipient block was placed in an oven at 37°C for two minutes, in order to attach the cores to the surrounding paraffin. Each TMA also contained internal controls, including healthy tissue (skin epithelia, normal cervical

tissue and colon polyps) and tumor tissue (breast, colon, and ovarian cancer). In total 7 TMAs, each containing approximately 200 cores, were constructed.

Immunohistochemistry

For immunohistochemistry, 3 μm sections were cut from the TMA. These sections were mounted on amino-propyl-ethoxy-silan (APES, Sigma-Aldrich, Diesenhofen Germany)-coated glass slides. Details of the antibodies used for immunohistochemistry and methods for antigen retrieval are summarized in Table 1. For antibody detection the avidin-biotin-peroxidase method was used for all, except pAKT. For pAKT staining the EnVision horseradish peroxidase system (Dako, Copenhagen, Denmark) was used. Slides were deparaffinized in xylene and rehydrated in ethanol. Endogenous peroxidase was blocked by incubation with 0.3% hydrogen peroxidase for 30 minutes. Staining was visualized by 3'3-diaminobenzidinetetrahydrochloride and counterstaining was performed with hematoxylin.

Table 1 – Antibodies used for immunohistochemical staining

Antigen	Antigen retrieval	Clone	Company	Dilution	Incubation time
EGFR	Proteinase K 0.1%, 30 minutes	111.6	Neomarkers†	1:200	60 minutes (RT¶)
pEGFR	EDTA (pH 8)*	1H12	Cell Signaling‡	1:200	60 minutes (RT¶)
PTEN	Citrate (pH 6)*	6H2.1	Cascade§	1:100	60 minutes (RT¶)
pAKT 1/2	Citrate (pH 6)*	736E11	Cell Signaling‡	1:50	overnight (4ºC)
pERK 1/2	Citrate (pH 6)*	20G11	Cell Signaling‡	1:50	overnight (4ºC)

*Sections were boiled in a microwave for 15 minutes
†Neomarkers, Lab Vision Corporation, Fremont, USA
‡Cell signaling, Danvers, USA
§Cascade Bioscience, Winchester, USA
¶Room temperature

Evaluation of staining

Staining intensity was semi-quantitatively scored as negative (0), weak positive (1+), positive (2+), and strong positive (3+). Also the percentage of positive cells was recorded. In case of differences between cores, scores were averaged for statistical analyses. Tumors were considered positive for EGFR in case of $\geq$10% membranous staining (22). pAKT and pERK staining were considered as positive if >10% of tumor cells showed positive (2+) cytoplasmic and/or nuclear staining (23). Positive staining of PTEN was defined as >10% cytoplasmic staining (16). Positive pEGFR staining was defined as at

least weak positive (1+) cytoplasmic staining, as the activated EGFR is internalized (24). Scoring was performed by two independent observers (MGN, KAH), without knowledge of clinical data. A concordance of more than 90% for all stainings was found. The discordant cases were reviewed and scores were reassigned on consensus of opinion. Only patients with at least two representative cores were included in the analysis.

Statistical analysis

Statistical analysis was performed with SPSS 16.0 for Windows (SPSS Inc., Chicago, IL). Differences in age were compared with the Student's t test. Other baseline characteristics, as well as associations between stainings, were compared with the Pearson's chi-square test. Associations between the presence of positive immunostaining and clinicopathological characteristics were assessed in logistic regression models, where immunostaining was used as dependent factor and the clinicopathological characteristics were used as independent factors. To determine factors involved in the presence of poor response to (chemo)radiation (model II), response to (chemo)radiation (as dependent factor) was evaluated in relation to clinicopathological factors and protein expression (as independent factors) with logistic regression analysis. Because treatment modality (radiotherapy vs. chemoradiation) is not a patient / tumor-dependent factor, but a time-dependent factor, as standard treatment changed over time from radiotherapy alone to chemoradiation it was included in multivariate analysis. Factors with a *P* value >0.10 were excluded stepwise in multivariate analysis; in the final step, only factors with a *P* value <0.05 were included. Disease-specific survival (DSS) was defined as the period from diagnosis to death as a consequence of cervical cancer, or last follow-up visit alive or death from another cause. Overall survival (OS) was defined as the time from diagnosis to death of any cause or last follow-up visit alive. Survival curves were generated using the Kaplan-Meier method, with evaluation of the differences by the Mantel-Cox log rank test. Differences in locoregional disease-free (model I), DSS and OS according to clinicopathological characteristics and protein expression were analyzed using Cox regression analyses. As chemoradiation is a time-dependent factor, multivariate analyses were adjusted for treatment modality. Variables with a *P* value >0.10 in univariate analysis were excluded stepwise in multivariate analysis; in the final step, only factors with a *P* value <0.05 were included. *P* values of <0.05 were considered statistically significant.

Results

Patient and tumor characteristics

From January 1980 to December 2006 489 patients were diagnosed with cervical cancer and primarily treated with (chemo)radiation. In 375 cases (77%) sufficient pretreatment tissue was available for TMA construction. The baseline characteristics of the 114 patients from whom no tumor tissue could be obtained differed from the study population, as they had more often advanced stage (≥IIb) disease (P<0.001). The other baseline characteristics were comparable (data not shown). Clinicopathological characteristics of patients included in this study are summarized in Table 2. Median follow-up time was 3.4 years (range 0.1 – 18.3) for all patients. For patients still alive at last follow-up median follow-up time was 6.0 years (range 0.5 – 18.3). Primary radiotherapy was given to 189 (50%) patients, whereas (chemo)radiation was given to 186 (50%) patients. The only difference in baseline characteristics between these two groups was that patients primarily treated with chemoradiation were younger (median age 46.8 vs. 64.8; P<0.001). Biopsies taken 8-10 weeks after completion of primary treatment or hysterectomy specimens of 279/375 (74%) patients were available to evaluate response to therapy. The patients who did not underwent biopsy or hysterectomy after therapy were significantly older than patients who did (median age 69.8 vs. 50.6; P<0.001).

Clinicopathological factors in relation to staining of EGFR, pEGFR, and pAKT

Immunohistochemistry was performed for EGFR, pEGFR, PTEN, pAKT, and pERK. The proportion of patients with less than 2 representative tissue cores varied from 1.6 – 5.1%. Fig. 1 shows a representative negative and positive tumor for each staining. Healthy cervical epithelium showed weak positive membranous EGFR and cytoplasmic pEGFR expression. PTEN stained positive and pERK stained weakly positive in the cytoplasm as well as in the nuclei, while pAKT was negative in cervical epithelium. Positive EGFR staining was present in 129/365 (35.3%), positive pEGFR staining in 71/361 (19.7%), positive PTEN staining was present in 126/369 (34.1%) tumors, positive pERK staining in 104/356 (29.2%), and positive pAKT staining in 15/364 (4.1%) of tumors. pEGFR staining was positively related to PTEN (P=0.001) and to pERK staining (P=0.004). EGFR positivity was inversely related to PTEN (P=0.011). No other associations were found (data not shown).

Table 2 – Patient and tumor characteristics

Age at diagnosis	**n=375**	
Median	54	
Range	21 – 91	
FIGO stage	*n*	*%*
Ib1	42	11%
Ib2	27	7%
IIa	51	14%
IIb	179	48%
IIIa	11	3%
IIIb	51	14%
IVa	14	4%
Histology		
Squamous	311	83%
Adenocarcinoma	52	14%
Other	12	3%
Differentiation grade		
Good / moderate	223	59%
Poor	128	34%
Unknown	24	6%
Lymphangioinvasion		
No	248	66%
Yes	54	14%
Unknown	73	19%
Tumor diameter		
0 – 4 cm	99	26%
≥4 cm	238	63%
Unknown	38	10%

Positive EGFR staining was less frequently observed in adenocarcinoma than in squamous cell carcinoma (OR=0.19; 95%CI=0.08 – 0.47; *P*<0.001) (Table 3). pEGFR positivity was related to high tumor stage (≥IIb) (OR=2.00; 95%CI=1.07 – 3.70; *P*=0.029), while poor differentiation (OR=0.39; 95%CI=0.21 – 0.75; *P*=0.004) and lymphangioinvasion (OR=0.20; 95%CI=0.06 – 0.66; *P*=0.008) were less frequently observed in pEGFR positive patients. Positive pAKT staining increased with age (OR=1.04; 95%CI=1.00 – 1.08; *P*=0.027). No associations were found for PTEN and pERK staining and any clinicopathological characteristic. As our specimens were collected over a long time period we also analyzed i.e. EGFR expression in carcinomas of the patients diagnosed before 1997 vs. those after 1997 (in 1997 the formula of formalin was slightly changed). We found no significant differences between the frequency of positivity before (59/163) and after 1997 (70/202), indicating that expression is not affected by the storage time of the tissue blocks in this study.

Figure 1 – Representative immunostained tumor tissue for EGFR, pEGFR, PTEN, pAKT, and pERK at 400x magnification.

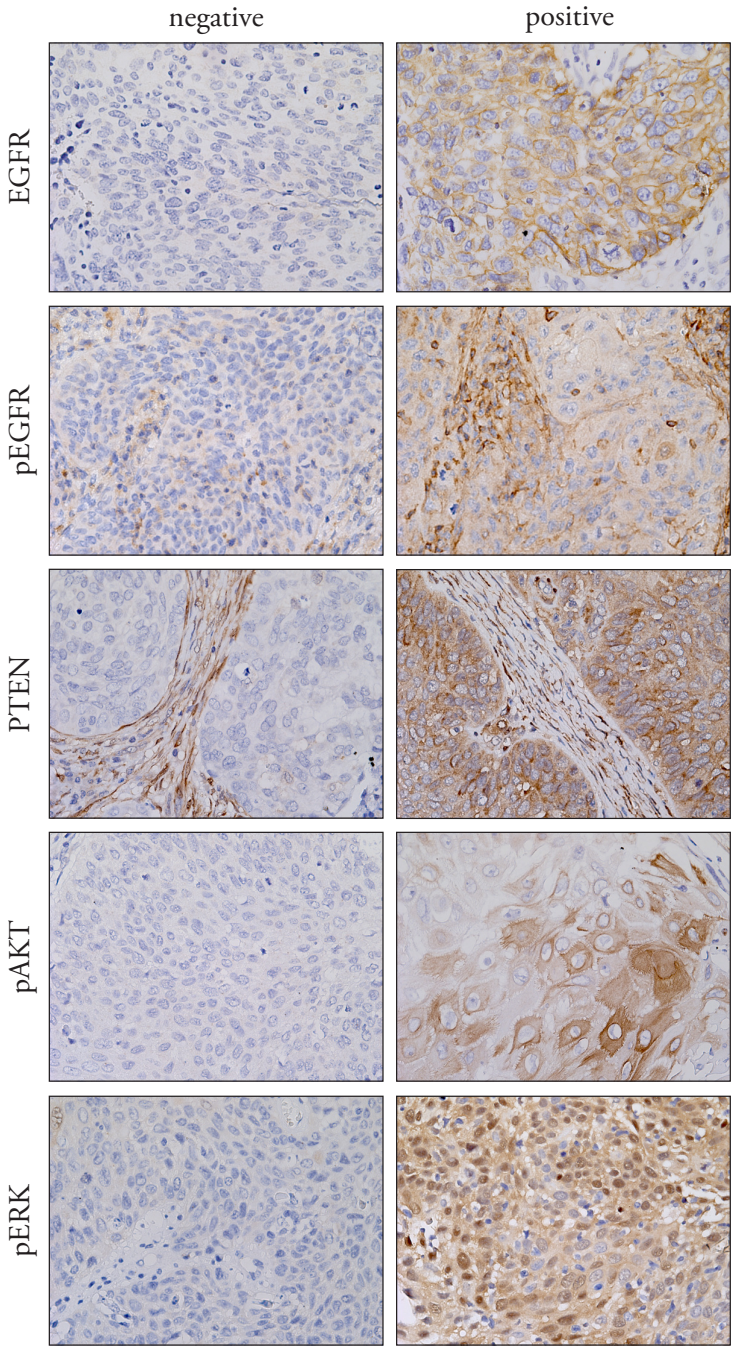


Table 3 – Relation between immunostaining and clinicopathological factors

	EGFR -		**EGFR +**		**EGFR +**		
	n/total	*%*	*n/total*	*%*	*OR**	*(95%CI)†*	*P value*
Age (continuous)					1.00	(0.99 – 1.02)	0.717
Age ≥54	121/236	51%	66/129	51%			
Stage ≥IIb	151/236	64%	95/129	74%	1.57	(0.98 – 2.52)	0.061
Adenocarcinoma	46/226	20%	6/127	5%	**0.19**	**(0.08 – 0.47)**	**<0.001**
Poor differentiation	87/226	38%	39/117	33%	0.80	(0.50 – 1.28)	0.348
Lymphangioinvasion	30/189	16%	23/105	22%	1.49	(0.81 – 2.72)	0.199
Tumor diameter ≥4 cm	141/209	67%	90/119	76%	1.50	(0.90 – 2.49)	0.120
	pEGFR -		**pEGFR +**		**pEGFR +**		
	n/total	*%*	*n/total*	*%*	*OR**	*(95%CI)†*	*P value*
Age (continuous)					1.02	(1.00 – 1.03)	0.071
Age ≥54	144/290	50%	42/71	59%			
Stage ≥IIb	189/290	65%	56/71	79%	**2.00**	**(1.07 – 3.70)**	**0.029**
Adenocarcinoma	38/279	14%	14/71	20%	1.56	(0.79 – 3.07)	0.200
Poor differentiation	108/271	40%	14/68	21%	**0.39**	**(0.21 – 0.74)**	**0.004**
Lymphangioinvasion	50/234	21%	3/59	5%	**0.20**	**(0.06 – 0.66)**	**0.008**
Tumor diameter ≥4 cm	182/263	69%	46/61	75%	1.36	(0.72 – 2.59)	0.340
	PTEN -		**PTEN +**		**PTEN +**		
	n/total	*%*	*n/total*	*%*	*OR**	*(95%CI)†*	*P value*
Age (continuous)					1.01	(1.00 – 1.03)	0.085
Age ≥54	121/243	50%	69/126	55%			
Stage ≥IIb	168/243	69%	82/126	65%	0.83	(0.53 – 1.31)	0.429
Adenocarcinoma	33/237	14%	19/120	16%	1.16	(0.63 – 2.15)	0.629
Poor differentiation	82/229	36%	44/117	38%	1.00	(0.52 – 1.91)	1.000
Lymphangioinvasion	35/202	17%	17/96	18%	1.03	(0.54 – 1.94)	0.935
Tumor diameter ≥4 cm	160/225	71%	74/107	69%	0.91	(0.55 – 1.50)	0.716
	pAKT -		**pAKT +**		**pAKT +**		
	n/total	*%*	*n/total*	*%*	*OR**	*(95%CI)†*	*P value*
Age (continuous)					**1.04**	**(1.00 – 1.08)**	**0.027**
Age ≥54	175/349	50%	12/15	80%			
Stage ≥IIb	233/349	67%	12/15	80%	1.99	(0.55 – 7.20)	0.293
Adenocarcinoma	50/337	15%	0/15	0%	0.00	(0.00 – 0.00)	0.997
Poor differentiation	120/331	36%	3/11	27%	0.66	(0.17 – 2.53)	0.544
Lymphangioinvasion	49/282	17%	2/12	17%	0.95	(0.20 – 4.48)	0.949
Tumor diameter ≥4 cm	96/219	44%	12/13	92%	5.26	(0.67 – 41.03)	0.113

Table 3 – (continued)

	pERK -		pERK +		pERK +		
	n/total	*%*	*n/total*	*%*	*OR**	*(95%CI)†*	*P value*
Age (continuous)					1.01	(0.99 – 1.02)	0.357
Age ≥54	129/252	51%	54/104	52%			
Stage ≥IIb	170/252	67%	67/104	64%	0.87	(0.54 – 1.41)	0.581
Adenocarcinoma	34/241	14%	16/103	16%	1.12	(0.59 – 2.13)	0.731
Poor differentiation	89/236	38%	36/97	37%	0.97	(0.60 – 1.59)	0.918
Lymphangioinvasion	35/199	18%	16/87	18%	1.06	(0.55 – 2.03)	0.870
Tumor diameter ≥4 cm	159/233	68%	67/89	75%	1.42	(0.81 – 2.47)	0.218

*Odds ratio
†95% confidence interval

EGFR and pEGFR are associated with response to (chemo)radiation

Locoregional progression during treatment or locoregional recurrence in follow-up was observed in 100/364 (27%) patients (model I). The location of recurrence of 11 patients was unknown and therefore these patients were not included in the analysis. Furthermore, 45 patients with clinical progression or persistence of disease at examination after completion of primary treatment and 147 patients with complete disease eridication were identified (model II). 44 of 45 patients with clinical progression or persistence of disease indeed died of their residual locoregional disease. Table 4 summarizes the relation between response to (chemo)radiation, immunohistochemical staining of the 5 parameters and clinicopathological characteristics in the two models. Univariate analysis revealed that EGFR and pEGFR staining were related to poor response in both models and therefore these stainings were included in multivariate analysis. In model I, positive staining of EGFR and pEGFR were independent predictors of poor response to therapy (EGFR: HR=1.84; 95%CI=1.20 – 2.82; *P*=0.005; pEGFR: HR=1.71; 95%CI=1.11 – 2.66; *P*=0.016), as confirmed by an even stronger relation between EGFR and pEGFR and response in model II analyzing the most extreme groups with respect to response only. Furthermore, simultaneous positive staining of both EGFR and pEGFR (n=21) was also significantly associated with response to (chemo)radiation in both univariate models (data not shown).

Table 4 – Response to (chemo)radiation

Model I: Cox regression analysis for time to clinical locoregional progression of disease during treatment or to locoregional recurrence after treatment (A), and model II: logistic regression analysis for patients with clinical progression or persistence of disease after treatment vs. patients with complete disease eradication (B).

A)	**Model I**	**Univariate**			**Multivariate***		
	n=364	*HR* †	*(95%CI)*§	*P value*	*HR* †	*(95%CI)*§	*P value*
	Age	1.01	(0.99 – 1.02)	0.268	¶		
	Stage ≥IIb	**2.54**	**(1.51 – 4.29)**	**<0.001**	**2.66**	**(1.52 – 4.66)**	**0.001**
	Adenocarcinoma	**1.72**	**(1.06 - 2.79)**	**0.027**	**2.07**	**(1.24 - 3.44)**	**0.005**
	Poor differentiation	1.04	(0.69 – 1.57)	0.861	¶		
	Lymphangioinvasion	0.95	(0.53 – 1.69)	0.859	¶		
	Tumor diameter ≥4 cm	**2.15**	**(1.27 - 3.64)**	**0.004**	•		
	EGFR positive	**1.77**	**(1.19 - 2.63)**	**0.005**	**1.84**	**(1.20 - 2.82)**	**0.005**
	pEGFR positive	**2.06**	**(1.35 - 3.15)**	**0.001**	**1.71**	**(1.11 - 2.66)**	**0.016**
	PTEN positive	0.73	(0.47 – 1.13)	0.162	¶		
	pAKT positive	0.67	(0.21 – 2.11)	0.495	¶		
	pERK positive	1.14	(0.74 – 1.74)	0.552	¶		
B)	**Model II**	**Univariate**			**Multivariate***		
	n=192	*OR* ‡	*(95%CI)*§	*P value*	*OR* ‡	*(95%CI)*§	*P value*
	Age	1.01	(0.99 – 1.04)	0.241	¶		
	Stage ≥IIb	**2.93**	**(1.27 – 6.64)**	**0.011**	•		
	Adenocarcinoma	**4.48**	**(1.83 – 10.98)**	**0.001**	**8.96**	**(2.99 – 26.85)**	**<0.001**
	Poor differentiation	0.86	(0.41 – 1.77)	0.678	¶		
	Lymphangioinvasion	1.64	(0.70 – 3.88)	0.258	¶		
	Tumor diameter ≥4 cm	**2.95**	**(0.16 - 7.53)**	**0.024**	•		
	EGFR positive	**3.28**	**(1.63 - 6.61)**	**0.001**	**6.08**	**(2.39 – 15.47)**	**<0.001**
	pEGFR positive	**2.86**	**(1.31 - 6.22)**	**0.008**	**4.06**	**(1.58 – 10.43)**	**0.004**
	PTEN positive	1.13	(0.56 – 2.26)	0.731	¶		
	pAKT positive	0.51	(0.06 – 4.33)	0.535	¶		
	pERK positive	1.13	(0.52 – 2.43)	0.759	¶		

*Adjusted for treatment modality
†Hazard ratio
‡Odds ratio
§95% confidence interval
¶Not included in multivariate analysis
•Not included in the final step of the multivariate analysis

Positive EGFR staining is related to poor prognosis

Positive immunostaining of EGFR and pEGFR were also related to DSS and OS in univariate analysis (Table 5). During the follow-up period 195/375 (52%) patients died,

of which 151 died of cervical cancer. The 5-year DSS rate was 53% in EGFR positive patients vs. 63% in EGFR negative patients and 50% in pEGFR positive patients vs. 60% in pEGFR negative patients (Fig. 2A and 2B). The 5-year OS rate was 47% in EGFR positive patients vs. 55% in EGFR negative patients and 48% in pEGFR positive patients vs. 53% in pEGFR negative patients (Fig. 2C and 2D). In multivariate Cox regression analysis for DSS including stage and histology, positive EGFR staining was independently related to poor DSS (HR=1.54; 95%CI=1.09 – 2.17; *P*=0.014) (Table 5). The relation between EGFR and OS was borderline significant (HR=1.33; 95%CI=0.99 – 1.77; *P*=0.058), and therefore EGFR was finally excluded from the stepwise multivariate analysis. pEGFR was not related to DSS (HR=1.30; 95%CI=0.86 – 1.98; *P*=0.216) and OS (HR=1.15; 95%CI=0.78 – 1.68; *P*=0.447) in multivariate analysis. Finally, simultaneous positive staining of both EGFR and pEGFR (n=21) was also significantly associated with DSS and OS in univariate analysis (data not shown).

Discussion

Our study in a large, well-documented series of consecutive cervical cancer patients primarily treated with (chemo)radiation, reveals that EGFR immunostaining is associated with poor DSS (HR=1.54; 95%CI=1.09 – 2.17; *P*=0.014). Furthermore, this study is the first to report that positive immunostaining of EGFR (HR=1.84; 95%CI=1.20 – 2.82; *P*=0.005) and pEGFR (HR=1.71; 95%CI=1.11 – 2.66; *P*=0.016) predict poor response to (chemo)radiation in cervical cancer, independent of stage, histology, and treatment modality. In our study, response to (chemo)radiation was defined in two different ways. The relation of both EGFR (OR=6.08; 95%CI=2.39 – 15.47; *P*<0.001) and pEGFR (OR=4.06; 95%CI=1.58 – 10.43; *P*=0.004) with response was the strongest in our model with the highest contrast in radiosensitivity (model II), supporting the idea that indeed EGFR and pEGFR are associated with a poor response to (chemo)radiation in cervical cancer. Interestingly, in this model stage was not an independent prognostic factor for poor response, while it is a strong prognostic factor for survival. However, response to (chemo)radiation is a different phenomenon, not necessarily related to stage, but to a variety of (cell biological) factors, such as hypoxia, etc., but also EGFR and pEGFR expression. An increased staining of EGFR has also been shown to be involved in poor response to radiotherapy in other malignancies, for example head & neck squamous cell cancer (18,19). The relation between EGFR and response to radiotherapy might be explained by the fact that EGFR is involved in DNA-double strand break re-

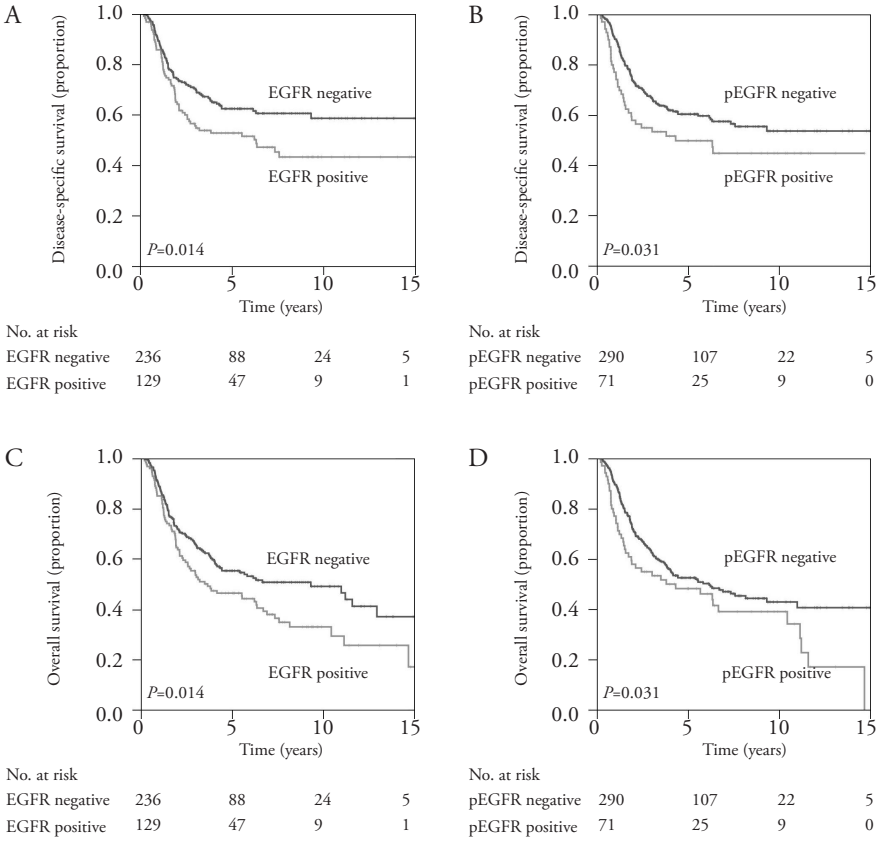
Table 5 – Results of Cox regression analysis for disease-specific death and death from any cause

Disease-specific death n=375	**Univariate** *HR* †	*(95%CI)*§	*P value*	**Multivariate*** *HR* †	*(95%CI)*§	*P value*
Age	1.00	(0.99 – 1.01)	0.596	¶		
Stage ≥IIb	**2.35**	**(1.56 - 3.54)**	**<0.001**	**2.56**	**(1.67 – 3.93)**	**<0.001**
Adenocarcinoma	**1.54**	**(1.02 – 2.32)**	**0.040**	**1.71**	**(1.11 – 2.63)**	**0.014**
Poor differentiation	1.24	(0.89 – 1.72)	0.211	¶		
Lymphangioinvasion	1.10	(0.70 – 1.72)	0.675	¶		
Tumor diameter ≥4 cm	**2.06**	**(1.35 – 3.13)**	**0.001**	•		
EGFR positive	**1.50**	**(1.08 – 2.08)**	**0.015**	**1.54**	**(1.09 – 2.17)**	**0.014**
pEGFR positive	**1.51**	**(1.04 – 2.20)**	**0.032**	•		
PTEN positive	0.80	(0.57 – 1.14)	0.222	¶		
pAKT positive	0.59	(0.22 – 1.58)	0.293	¶		
pERK positive	1.18	(0.83 – 1.68)	0.350	¶		
Death from any cause n=375	**Univariate** *HR* †	*(95%CI)*§	*P value*	**Multivariate*** *HR* †	*(95%CI)*§	*P value*
Age	**1.01**	**(1.01 – 1.02)**	**0.002**	•		
Stage ≥IIb	**1.97**	**(1.39 – 2.78)**	**<0.001**	**1.97**	**(1.39 – 2.78)**	**<0.001**
Adenocarcinoma	1.21	(0.81 – 1.79)	0.354	¶		
Poor differentiation	1.15	(0.85 – 1.55)	0.355	¶		
Lymphangioinvasion	1.12	(0.75 – 1.66)	0.590	¶		
Tumor diameter ≥4 cm	**1.69**	**(1.19 – 2.39)**	**0.003**	•		
EGFR positive	**1.43**	**(1.07 – 1.91)**	**0.016**	•		
pEGFR positive	**1.43**	**(1.02 – 2.00)**	**0.039**	•		
PTEN positive	0.87	(0.64 – 1.19)	0.383	¶		
pAKT positive	0.71	(0.33 – 1.54)	0.390	¶		
pERK positive	1.15	(0.83 – 1.58)	0.401	¶		

*Adjusted for treatment modality
†Hazard ratio
§95% confidence interval
¶Not included in multivariate analysis
•Not included in the final step of the multivariate analysis

pair (25). Radiation-induced EGFR signaling activates the PI3K/AKT pathway, resulting in DNA-double strand break repair by interaction with DNA-dependent protein kinases (26). Another explanation might be that radiation activates EGFR signaling, even in the absence of ligand binding, for example by increasing TGF-α expression, which can activate EGFR (27). As a consequence, this activation of the downstream signaling cascades causes inhibition of apoptosis and promotion of cell proliferation (4). Therefore, carcinomas with increased levels of EGFR or pEGFR might activate

Figure 2 – Survival curves. Kaplan-Meier curves for the relation of EGFR and pEGFR immunostaining with disease-specific (A, B) and overall survival (C, D).



this EGFR signaling pathway more efficiently, resulting in a decreased local control.

Because of its apparent involvement in response to radiotherapy, EGFR targeted therapy has recently been implemented as a new therapeutic strategy in various malignancies (reviewed in (28)). However, the relation between EGFR protein expression and response to EGFR inhibitors is questionable, as colorectal cancer patients without detectable EGFR protein expression did respond to treatment with cetuximab (29,30). Various EGFR related biomarkers were found to predict response to treatment with EGFR inhibitors. For instance, in colorectal cancer and non small cell lung cancer *KRAS* mutations are associated with resistance to EGFR inhibitors (31), while specific *EGFR* mutations and high copy numbers of the *EGFR* gene predict a better response in non-small cell lung cancer (32,33). In cervical cancer neither *EGFR* mutations (34), nor *EGFR* gene amplification (35), and only a few (0-8%) *KRAS* mutations (36-39)

have been observed. These data combined with the relation that we find for EGFR and pEGFR immunostaining and poor response to (chemo)radation suggest that the addition of EGFR inhibitors to standard chemoradiation should be evaluated in advanced stage cervical cancer patients. Up till now, in cervical cancer only a single phase II study using gefitinib, an EGFR tyrosine kinase inhibitor (EGFR TKI), as monotherapy for recurrent cervical cancer was reported recently with modest response rates (40). Clinical trials with cetuximab in addition to (chemo)radiation in the treatment of locally advanced cervical cancer are ongoing (http://www.cancer.gov/clinicaltrials, NCT00104910).

No relation was found between PTEN, pAKT, and pERK and response to (chemo)radiation. Positive PTEN staining was observed in 34.1% of tumors, which is lower than previously reported (16,41,42). This might be due to differences in study populations, as other studies mainly focussed on early stage cervical cancer and positive PTEN staining decreases in more advanced stage disease (16). In our study pAKT was only positive in 4.1% of tumors. In previous studies pAKT immunostaining was observed in 29-94% (13,14,43), although the same antibody and protocol for immunostaining were used. pAKT staining was not related to response to therapy nor to survival, possibly due to the relatively small number of positive cases in our study. To our knowledge, this is the first study investigating pERK protein expression in cervical cancer. Activated ERK was not related to response to therapy, nor to survival.

In conclusion, our study indicates that EGFR and pEGFR immunostaining are independent markers for poor response to (chemo)radiation and EGFR immunostaining is an independent poor prognostic factor for DSS. In advanced stage cervical cancer patients, the apparent involvement of EGFR in response to (chemo)radiation presents the EGFR pathway as a promising therapeutic target in already ongoing clinical trials, in which EGFR inhibitors are combined with standard chemoradiation in cervical cancer patients.

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Poor response to (chemo)radiation is related to high levels of pATM in advanced stage cervical cancer patients

Manuscript in preparation

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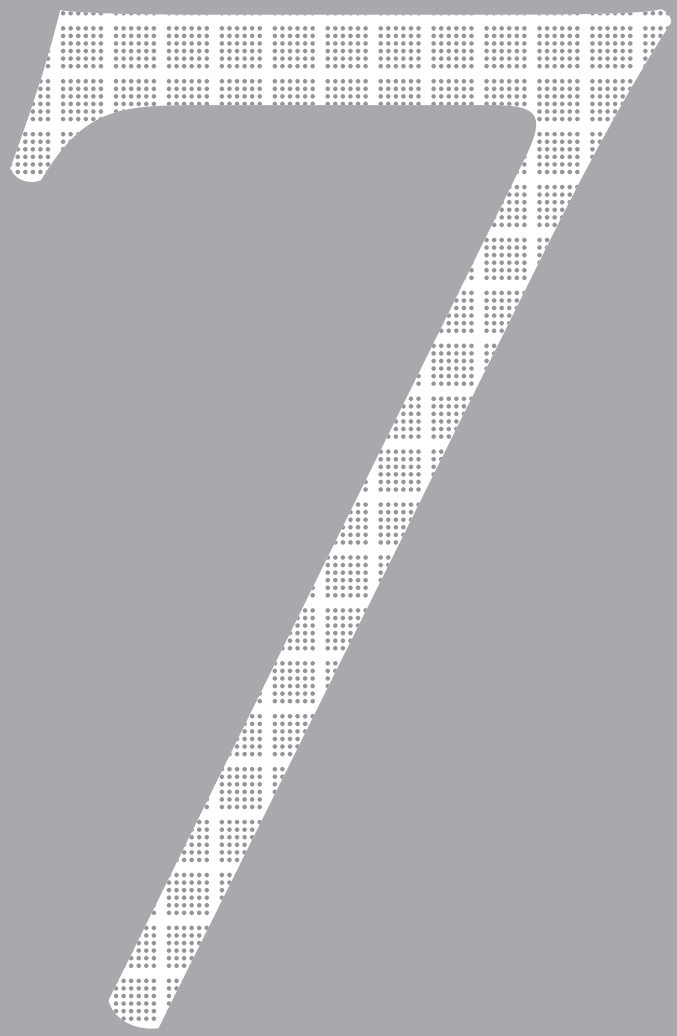


Abstract

Introduction: Chemoradiation is the standard of care for advanced stage cervical cancer patients. During chemoradiation, DNA double strand breaks (DSBs) are introduced to cause sufficient genotoxicity for cell death induction. Central in the response to DSBs is the ataxia telangiectasia mutated (ATM) protein, which in event of DNA damage is activated through auto-phosphorylation (pATM). Subsequently, a signaling cascade ultimately leads to DSB-repair. In this study, we examined the phosphorylation status of ATM in relation to response to (chemo)radiation and survival in a large, well-documented series of cervical cancer patients.

Patients and methods: Pretreatment tissue samples of 375 consecutive FIGO stage Ib to IVa cervical cancer patients treated with (chemo)radiation between January 1980 and December 2006 were collected. Clinicopathologic and follow-up data were prospectively obtained during standard treatment and follow-up. Protein expression of pATM and non-phosphorylated ATM (nATM) was assessed by immunohistochemistry on tissue microarrays. Response to chemoradiation was measured by locoregional disease-free survival.

Results: High immunostaining (defined as positive staining in $\geq$75% of the nuclei) against nATM and pATM was observed in 15% (n=53) and 52% (n=183), respectively. High nATM immunostaining was related to poor response to (chemo)radiation (HR=1.834; 95%CI=1.137 – 2.958; *P*=0.013). Moreover, high pATM immunostaining was related to poor response to (chemo)radiation in both univariate (HR=1.868; 95%CI=1.219 – 2.862; *P*=0.004) and multivariate analysis (HR=1.643; 95%CI=1.015 – 2.658; *P*=0.043), as well as shorter disease-specific survival (HR=1.488; 95%CI=1.065 – 2.081; *P*=0.020).

Conclusion: High expression of nATM and pATM is related to poor response to (chemo)radiation in advanced stage cervical cancer patients. Inhibition of ATM activity could serve as a therapeutic target in future anti-cancer treatment.

Introduction

The standard of care for advanced stage cervical cancer patients has shifted over the last decade from radiotherapy alone to platinum-based chemoradiation (1). Despite this shift in treatment modality, the 5-year survival is still around 66% (2). Chemoradiation introduces DNA double strand breaks (DSBs), aimed to cause sufficient genotoxicity to induce cell death, presumably through apoptosis (3-5). Relevant for the response to radiotherapy are proteins that are involved in signaling and repairing DSBs. The ataxia telangiectasia mutated (ATM) protein is a serine/threonine specific-protein kinase and a key protein involved in DSB signaling (6). Briefly, upon DSB DNA damage a mediator complex consisting of MRE11, RAD50 and NBS1 (MRN) is recruited to the breakage site (7-10). Subsequent (auto-)phosphorylation of ATM at Serine 1981 (Ser1981) results in active monomeric ATM (pATM) proteins (11). Upon activation, ATM phosphorylates a variety of targets including: H2AX, MDC1, CHK2, 53BP1, BRCA1 and many others, resulting in cell cycle checkpoint activation and DNA repair (8,12-14).

Previous investigations of the role of ATM in response to radiotherapy in cervical cancer have been limited to cell lines only (15-17). In these studies, downregulation of ATM, through RNA interference and targeted drugs, resulted in the radiosensitization of cervical cancer cells. Furthermore, downregulation of ATM in other malignancies, such as head & neck (18), gliomas (19), breast (17,20), lung (21), and prostate cancer (22), provided similar results, predominantly obtained in cell lines. In addition, patients suffering from Ataxia Telangiectasia (AT), a rare disorder in which the ATM is mutated and dysfunctional, also exhibit a sensitive phenotype to radiotherapy (6).

Expression of ATM, both in its native and activated state, may play an important role in predicting the response to radiotherapy in cervical cancer patients. Therefore, the aim of this study was to investigate the level of expression of ATM, both in its unphosphorylated and phosphorylated (Ser1981) state, in relation to response to (chemo)radiation and survival in a large, well-documented, consecutive series of cervical cancer patients, primarily treated with (chemo)radiation.

Patients and methods

Patients and treatment modalities, institutional review board approval and tissue microarray construction

Our group has established a large database of biological material and follow-up data from cervical cancer patients treated at the University Medical Center Groningen (UMCG) or collaborating hospitals. Routine patient follow-up time was at least five years or until January 2009. Staging of patients was according to FIGO guidelines. This patient cohort and corresponding treatments, review board approval and tissue microarray (TMA) construction has been previously described (23).

Evaluation of response to (chemo)radiation

Approximately 8-10 weeks after completion of (chemo)radiation either hysterectomy or biopsy was performed if a patient was (technically) classified as operable. Therefore, not all patients in our database underwent a post-treatment biopsy and/or hysterectomy. As a consequence, the response to (chemo)radiation could not be evaluated based on this parameter in all patients. Therefore, we evaluated response to (chemo)radiation retrospectively in two ways. First (Model I) the response to (chemo)radiation was determined based on locoregional disease-free survival. Locoregional disease-free survival is defined as the period from diagnosis until locoregional progression of disease during treatment or locoregional recurrence. Patients were excluded from the analysis, if the location of the recurrence was unknown. Secondly (Model II), response to (chemo)radiation was determined in two subsets of patients, with supposedly the largest difference in treatment response. In the first subset, patients with no residual tumor-material in their post treatment specimen and without locoregional recurrence during follow-up with a minimum of two years were selected. The second subset consists of patients with clinical evidence of disease progression during treatment or clinical evidence of disease persistence at examination after completion of primary treatment. Both models also have been described and used previously (23).

Immunohistochemical staining with antibodies against ATM and pATM

The TMAs were immunohistochemically stained with monoclonal antibodies against non-phosphorylated ATM (nATM; 1:5, 1h; Rabbit IgG, Epitomics, Clone Y170) and pATM (1:50, 1h; Rabbit IgG, Epitomics, Clone EP1890Y, S1981). The nATM antibody detects only non-phosphorylated ATM, whereas the pATM antibody recognizes

exclusively the phosphorylated (S1981) ATM product and therefore, both antibodies are mutually exclusive (24).

From our TMA-paraffin blocks, 3µm sections were cut and placed on amino-propyl-etoxy-silan (APES) coated glass slides. Antigen retrieval was achieved for the nATM antibody by 16h incubation at 80°C. For the pATM antibody, the antigen was retrieved using the microwave. Slides were deparaffinized using Xylene and subsequently rehydrated using a multistep process from ethanol to phosphate buffered saline (PBS). Endogenous peroxidase activity was blocked by incubating with 0.3% hydrogen peroxidase for 30 minutes. Both antibodies were detected using horse-radish-peroxidase (HRP) conjugated streptavidin with subsequent visualization using 3'3 – diaminobenzidine-tetrahydrochloride (AEC). Counterstaining was achieved using hematoxylin.

Evaluation of staining

Staining intensity was semi-quantitatively scored as negative (0), weak positive (1), positive (2), and strong positive (3). In addition, the percentage of positive cells per staining intensity was documented for each core. Since not only the amount of expression of either nATM or pATM (24), but also the amount of cells that are positive could play an important role in the response to (chemo)radiation, we have analyzed our data using two immunostaining scenarios. (1) Moderate immunostaining, which is defined as patients with positive nuclear immunostaining with an intensity of at least 2 present in at least 50% of tumor cells and (2) high immunostaining, which is defined as patients with positive nuclear immunostaining with at least 2 present in at least 75% of tumor cells. TMA evaluation was performed independently by two observers (MGN, FR) without prior knowledge of the clinical data. A concordance of more than 90% was found between both observers, for both immunostainings. Subsequent evaluation of disconcordant cases was performed to reach a consensus score. Only patients with at least two evaluable tumor cores were included for statistical analysis.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL). The student's t-test was used to analyze differences in age. Other baseline characteristics, as well as associations between immunostainings, were compared with the Pearson's χ^2 test. In addition, logistic regression models were used to evaluate positive staining and clinicopathological characteristics, with immunostaining being the dependent and the clinicopathological characteristics the independent factors. To identify

factors involved in response to (chemo)radiation, response to (chemo)radiation was evaluated in relation to clinicopathological factors (dependent) and immunostaining (independent) using Cox-regression analysis for Model I and logistic regression analysis in Model II. Disease-specific survival was defined as the time from diagnosis until the last follow-up alive or death due to other causes than cervical cancer or death due to cervical cancer. Overall survival was defined as the time from diagnosis until the last follow-up visit (alive) or death due to any cause. Survival was visualized using the Kaplan-Meier method and Mantel-Cox log rank test was used to evaluate the differences between these curves. Disease-specific survival and overall survival were analyzed using the Cox regression analysis. Since chemoradiation is a time-dependent factor and associated with a better survival, multivariate analyses were adjusted for treatment modality. Variables with a *P* value of <0.10 in univariate analysis were excluded stepwise in multivariate analysis; in the final step, only factors with a *P* value of <0.05 were included. *P* values <0.05 were considered statistically significant.

Results

Patients and treatment modalities and TMA construction

In the period from January 1980 until December 2006, 489 consecutive patients were diagnosed with cervical cancer and primarily treated with (chemo)radiation. Of 375 patients (77%), sufficient pretreatment tissue was available for TMA construction**.** The remaining 114 patients tissues (23%) that were excluded for TMA construction, differed significantly from the 375 selected cases based on their stage of disease (≥IIb; *P*<0.001). Other baseline characteristics were comparable between both groups (data not shown). Clinicopathological data of patients, included in this study, are summarized in Table 1. The mean follow-up time was 3.6 (range: 0.1 – 18.3) years for all patients. For patients who were still alive at time of their final follow-up median follow-up time was 6.0 years.

In 189 (50%) cases only radiotherapy was given, whereas 186 (50%) patients received chemoradiation. Patients who received chemoradiation were younger compared to patients who received RT alone (Median 46.8 vs. 64.8, *P*<0.001). All other baseline characteristics were comparable in both groups (data not shown).

Clinicopathological factors in relation to staining of ATM and pATM

Of the 375 patient samples present on the TMA, 350 (93.9%) cases for nATM and 349 (93.1%) cases for pATM were evaluable for statistical analysis. Fig. 1 depicts rep-

Table 1 – Patient characteristics

Age at diagnosis	**n=375**	
Median	54	
Range	21 – 91	
FIGO stage	*n*	*%*
Ib1	42	11%
Ib2	27	7%
IIa	51	14%
IIb	179	48%
IIIa	11	3%
IIIb	51	14%
IVa	14	4%
Histology		
Squamous	311	83%
Adenocarcinoma	52	14%
Other	12	3%
Differentiation grade		
Good / moderate	223	59%
Poor	128	34%
unknown	24	6%
Lymphangioinvasion		
No	248	66%
Yes	54	14%
unknown	73	19%
Tumor diameter		
0 – 4 cm	99	26%
≥4 cm	238	63%
unknown	38	10%

resentative cases for both stainings. Any positive nuclear staining (≥10% of intensity ≥1) for nATM was observed in 334 cases (95.4%) and for pATM in 344 cases (98.6%), indicating that ATM, regardless of phosphorylation state, is present in virtually all cases. Moderate and high nATM immunostaining was observed in 92 (26.3%) and 53 cases (15.1%) respectively, whereas moderate and high pATM immunostaining was observed in 258 (73.9%) and 183 (52.4%) cases, respectively. To exclude whether long-term storage has influenced immunostaining intensity in our patient samples, we tested if immunostaining intensities differed between time periods. No significant differences were found. This has also been shown for other markers in previous studies (23).

Figure 1 – Representative cases of immunostaining for pATM and nATM

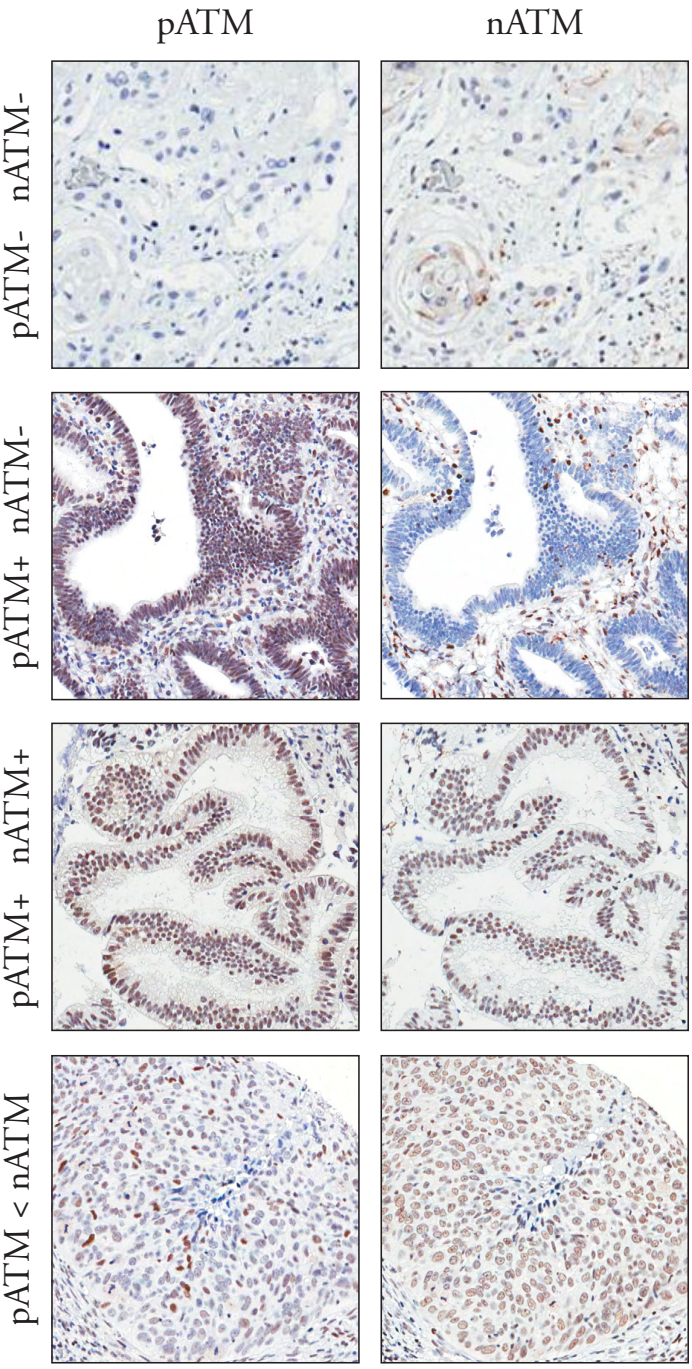


Table 2 – Relation between immunostaining and clinicopathological factors

	Moderate nATM			**High nATM**		
	OR *	*(95%CI)*†	*P value*	*OR* *	*(95%CI)*†	*P value*
Age	1.005	(0.990 – 1.021)	0.498	1.005	(0.987 – 1.024)	0.571
Stage ≥IIb	1.411	(0.834 – 2.389)	0.199	**2.027**	**(1.001 – 4.105)**	**0.050**
Adenocarcinoma	**3.516**	**(1.891 – 6.535)**	**<0.001**	**3.110**	**(1.544 – 6.266)**	**0.001**
Poor differentiation	0.875	(0.519 – 1.476)	0.617	1.083	(0.576 – 2.036)	0.803
Lymphangioinvasion	0.701	(0.330 – 1.487)	0.354	0.675	(0.269 – 1.693)	0.402
Tumor diameter ≥4 cm	1.035	(0.600 – 1.786)	0.902	1.337	(0.662 – 2.701)	0.418
	Moderate pATM			**High pATM**		
	OR *	*(95%CI)*†	*P value*	*OR* *	*(95%CI)*†	*P value*
Age	**0.974**	**(0.959 – 0.989)**	**0.001**	**0.981**	**(0.968 – 0.994)**	**0.006**
Stage ≥IIb	1.366	(0.830 – 2.246)	0.219	**1.851**	**(1.179 – 2.905)**	**0.007**
Adenocarcinoma	1.486	(0.709 – 3.114)	0.294	1.778	(0.954 – 3.311)	0.070
Poor differentiation	0.781	(0.472 – 1.293)	0.337	1.259	(0.799 – 1.983)	0.321
Lymphangioinvasion	0.721	(0.371 – 1.399)	0.333	0.715	(0.389 – 1.314)	0.280
Tumor diameter ≥4 cm	**1.743**	**(1.030 – 2.949)**	**0.038**	**1.848**	**(1.135 – 3.010)**	**0.014**

*Odds ratio
†95% confidence interval

Table 2 summarizes the relation between clinicopathological data and immunostaining. Both moderate and high nATM staining were related to adenocarcinoma (moderate nATM; OR=3.516; 95%CI=1.891 – 6.535; *P*<0.001) (high nATM; OR=3.110; 95%CI=1.544 – 6.266; *P*=0.001). In addition, high nATM immunostaining was related to tumor stage (≥IIb; OR=2.027; 95%CI=1.001 – 4.105; *P*=0.05). Moderate pATM immunostaining was related to tumor diameter (OR=1.743; 95%CI=1.030 – 2.949; *P*=0.038), whereas high pATM staining was related to tumor stage (≥IIb; OR=1.851; 95%CI=1.179 – 2.905; *P*=0.007), age (OR=0.981; 95%CI=0.968 – 0.994; *P*=0.006) and tumor diameter (≥4cm; OR=1.848; 95%CI=1.135 – 3.010; *P*=0.014).

Expression of nATM and pATM are related to poor response to (chemo)radiation

As described above, we assessed protein expression in relation to the response to (chemo)radiation retrospectively in two models. Table 3 summarizes the response to chemoradiation in relation to nATM and pATM immunostaining and clinicopathological factors for both models. In Model I, where treatment response is based on locoregional control, 364 patients (97.1%) were selected. The remaining 11 patients

Table 3 – Response to radiotherapy

Model I: Cox regression analysis for time to clinical locoregional progression of disease during treatment or until locoregional recurrence after treatment(A). Model II: logistic regression analysis for patients with clinical progression or persistence of disease after treatment vs. patients with complete disease eradication (B).

A)	**Model I**	**Univariate**			**Multivariate***		
	n=364	*HR* †	*(95%CI)*§	*P value*	*HR* †	*(95%CI)*§	*P value*
	Age	1.008	(0.995 – 1.020)	0.224	¶		
	Stage ≥IIb	**2.552**	**(1.514 – 4.299)**	**<0.001**	**2.702**	**(1.550 – 4.711)**	**<0.001**
	Adenocarcinoma	**1.712**	**(1.057 – 2.772)**	**0.029**	•		
	Poor differentiation	1.014	(0.672 – 1.530)	0.947	¶		
	Lymphangioinvasion	0.944	(0.532 – 1.678)	0.845	¶		
	Tumor diameter ≥4cm	**2.171**	**(1.284 – 3.671)**	**0.004**	•		
	Moderate nATM	**1.529**	**(0.998 – 2.343)**	**0.051**	•		
	High nATM	**1.834**	**(1.137 – 2.958)**	**0.013**	•		
	Moderate pATM	1.339	(0.817 – 2.194)	0.247	¶		
	High pATM	**1.868**	**(1.219 – 2.862)**	**0.004**	**1.643**	**(1.015 – 2.658)**	**0.043**
B)	**Model II**	**Univariate**			**Multivariate***		
	n=197	*OR* ‡	*(95%CI)*§	*P value*	*OR* ‡	*(95%CI)*§	*P value*
	Age	1.013	(0.990 – 1.036)	0.266	¶		
	Stage ≥IIb	**3.016**	**(1.315 – 6.921)**	**0.009**	**3.523**	**(1.334 – 9.303)**	**0.011**
	Adenocarcinoma	**4.655**	**(1.902 – 11.394)**	**0.001**	**3.840**	**(1.437 – 10.266)**	**0.007**
	Poor differentiation	0.852	(0.413 – 1.758)	0.665	¶		
	Lymphangioinvasion	1.630	(0.694 – 3.827)	0.262	¶		
	Tumor diameter ≥4cm	**3.013**	**(1.183 – 7.673)**	**0.021**	•		
	Moderate nATM	1.829	(0.880 – 3.799)	0.106	¶		
	High nATM	**3.075**	**(1.317 – 7.182)**	**0.009**	•		
	Moderate pATM	1.557	(0.661 – 3.669)	0.311	¶		
	High pATM	**2.600**	**(1.260 – 5.365)**	**0.010**	**2.355**	**(1.051 – 5.279)**	**0.038**

*Adjusted for treatment modality
†Hazard ratio
‡Odds ratio
§95% confidence interval
¶Not included in multivariate analysis
•Not included in the final step of the multivariate analysis

were excluded for analysis due to unknown locations of their recurrence. In this model, high nATM immunostaining was related to poor locoregional disease-free survival in univariate Cox regression analysis (HR=1.834; 95%CI=1.137 – 2.958; *P*=0.013). In addition, high pATM was also related to poor locoregional disease-free survival in univariate (HR=1.868; 95%CI=1.219 – 2.862; *P*=0.004) as well as multivariate analysis (HR=1.643; 95%CI=1.015 – 2.658; *P*=0.043).

In Model II, we analyzed our data in two subsets of patients with supposedly the highest contrast in treatment response. 152 patients with complete eradication of dis-

ease were identified as "Responder" and 45 patients who had clinical progression of disease at examination 8-10 weeks after completion of primary treatment, were identified as "Non-responder". Of these so-called Non-responders, 44 patients (98%) died due to residual locoregional disease. In this model, a relation between high nATM (OR=3.075; 95%CI=1.317 – 7.182; *P*=0.009) and poor response to treatment was found in univariate logistic regression analysis. Furthermore, also high pATM immunostaining was again related to response to (chemo)radiation in univariate (OR=2.600; 95%CI=1.260 – 5.365; *P*=0.010) as well as multivariate analysis (OR=2.355; 95%CI=1.051 – 5.297; *P*=0.038).

High immunostaining against pATM is related to poor prognosis

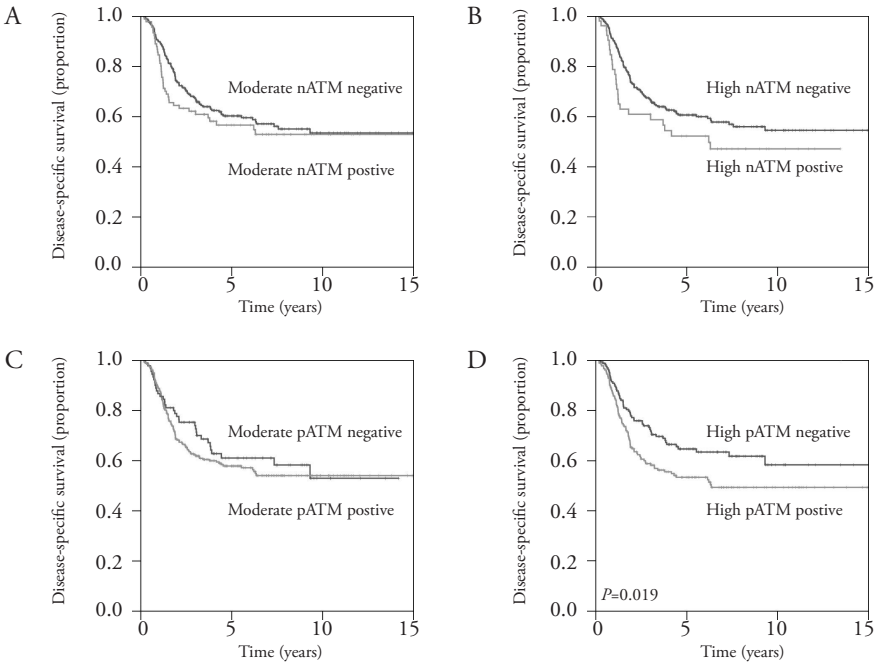
During routine follow-up, 195 of 375 (52%) of patients eventually died (overall survival). In 151 (77%) of these patients, death was cervical cancer related (disease-specific survival). Table 4 summarizes DSS in relation to nATM and pATM immunostaining and clinicopathological data. We found that high pATM immunostaining was related to worse disease-specific survival (HR=1.49; 95%CI=1.06 – 2.08; *P*=0.020) in univariate analysis. Fig. 2 depicts Kaplan-Meier survival curves for disease-specific survival in respect to nATM and pATM immunostaining. The log-rank *P* value for high pATM immunostaining was *P*=0.019.

Table 4 – Disease-specific survival

	Univariate			**Multivariate***		
	HR †	*(95%CI)*§	*P value*	*HR* †	*(95%CI)*§	*P value*
Age	1.002	(0.992 – 1.012)	0.693	¶		
Stage ≥IIb	**2.287**	**(1.526 – 3.429)**	**<0.001**	**2.016**	**(1.305 – 3.115)**	**0.002**
Adenocarcinoma	**1.536**	**(1.017 – 2.319)**	**0.041**	•		
Poor differentiation	1.251	(0.900 – 1.739)	0.183	•		
Lymphangioinvasion	1.151	(0.742 – 1.784)	0.531	¶		
Tumor diameter ≥4cm	**1.991**	**(1.316 – 3.014)**	**0.001**	**1.630**	**(1.055 – 2.520)**	**0.028**
Moderate nATM	1.197	(0.831 – 1.726)	0.334	¶		
High nATM	1.418	(0.926 – 2.170)	0.108	¶		
Moderate pATM	1.169	(0.795 – 1.718)	0.428	¶		
High pATM	**1.488**	**(1.065 – 2.081)**	**0.020**	•		

*Adjusted for treatment modality
†Hazard ratio
§95% confidence interval
¶Not included in multivariate analysis
•Not included in the final step of the multivariate analysis

Figure 2 – Survival curves. Kaplan-Meier curves for the relation of moderate (A) and high (B) nATM immunostaining, and moderate (C) and high (D) pATM immunostaining and disease-specific survival.



Discussion

To our knowledge this is the first time that ATM has been assessed by immunohistochemistry in both its native and phosphorylated state in a large, well-documented, series of cervical cancer patients primarily treated with (chemo)radiation. Our results strongly suggest involvement of nATM as well as pATM in the response to (chemo)radiation in advanced stage cervical cancer patients. We showed that high nATM immunostaining as well as high pATM immunostaining was related to poor response to (chemo)radiation in univariate analysis in both Model I, based on locoregional disease-free survival. Importantly, these relations were even more pronounced in our second analysis model (Model II), in which we selected two subsets of patients with supposedly the largest contrast in treatment response, indicating that there is an involvement of nATM and especially pATM in relation to poor (chemo)radiation response. Furthermore, we found that high pATM immunostaining was related to disease-specific survival.

Literature regarding relation of ATM expression in response to (chemo)radiation and survival in other malignancies is limited. Sarbia et al. found that ATM immuno-

staining was not associated with regression after neoadjuvant chemoradiation and overall survival in oesophageal squamous cell carcinomas (25). Another report regarding patients with early stage breast cancer, who underwent postoperative radiotherapy or adjuvant chemotherapy, also found no relation between ATM expression and response after radiotherapy (26). In addition, protein expression of ATM was not a prognostic factor in pancreatic cancer patients (27). However, in colon cancer, Grabsch *et al.* reported that positive immunostaining of ATM was related to good survival in large series of colorectal cancer patients primarily treated by surgery. Some of these patients also received adjuvant chemo- and/or radiotherapy, however these numbers were limited to conclude (28). Finally, mutations of *Atm* have been studied in breast cancer patients treated by adjuvant radiotherapy following conservative surgery, and presence of a mutation in the *Atm* gene was associated with poor metastases-free survival (29). The difference in results between these reports and our study could be explained by differences in treatment modality between and even within these studies. Moreover, our study focuses on the phosphorylation status of ATM (nATM and pATM), whereas most other studies focussed on expression of ATM, regardless of phosphorylation state. Importantly, our data is in line with previous *in vitro* investigations in various cancer cells lines, including cervical cancer, in which downregulation of ATM resulted in enhanced response to radiotherapy (15-20).

The important role that ATM has in signaling DNA damage could serve as an explanation of our results. One could speculate, that when a tumor has a majority of cells with high amounts of (p)ATM readily present in nucleus prior to treatment, that these cells could be more efficient in signaling DNA damage and subsequent reparation. As a consequence, this tumor has then a better chance of survival after (chemo)radiation, which leads to poor response to (chemo)radiation in patients.

Based on our results, specific targeting of the ATM kinase activity could be an option for future therapy for cervical cancer patients who have high levels of ATM present before start of therapy. Previous investigations in cervical cancer cell lines showed that inhibition of ATM by either RNA interference or targeted drug application result in enhanced sensitivity to radiotherapy (15,16). Furthermore, other investigations regarding enhanced radiosensitivity of cells after inhibition of ATM have been reported in multiple malignancies (15-21). At present, no FDA approved ATM inhibitor is available. However, ATM-inhibitors, like KU-60019, an improved version of KU-55933, has been shown to effectively inhibit the radiation induced phosphorylation of key downstream targets of ATM and results in sensitization of glioma cells *in vitro* (30).

In conclusion, we have shown that phosphorylation of ATM predicts poor response

to (chemo)radiation in advanced stage cervical cancer patients. Therefore, specific inhibiting ATM phosphorylation or inhibition of the kinetic activity of ATM could provide an additional target for the improvement of the response to (chemo)radiation in advanced stage cervical cancer patients.

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Summary and future perspectives

Summary

Cervical cancer is a disease with severe morbidity and a high mortality rate in women worldwide (1,2). New treatment modalities are urgently needed to improve survival rates, without causing an increase of short- and long-term side effects. Treatment of early stage cervical cancer patients (International Federation of Gynecology and Obstetrics [FIGO] stage Ia-IIa) often consists of radical hysterectomy and pelvic lymph node dissection. Adjuvant radiotherapy or chemoradiation is administered in case of poor prognostic factors, with pelvic lymph node metastases as the most important prognostic factor (3,4). Standard treatment of locally advanced cervical cancer (FIGO stage Ib2, IIb-IVa) is concurrent cisplatin-based chemoradiation, resulting in a 5-year overall survival rate of 66% (5). Thus, choice of treatment is nowadays mostly based on well-known clinicopathological factors, such as FIGO stage and presence of lymph node metastases. Cell biological markers could also be of potential clinical relevance. Discovery of cell biological markers associated with response to treatment or prognosis might be helpful in identification of new targets for therapeutic intervention. In addition, cell biological markers could be helpful in predicting presence of pelvic lymph node metastases, response to chemoradiation and prognosis in the individual patient (6).

In this thesis, cell biological markers and pathways associated with lymph node metastases, response to (chemo)radiation, and prognosis in cervical cancer were investigated.

In **chapter 2**, a systematic review of well-documented studies on prognostic and predictive cell biological markers, comprising $\geq$50 cervical cancer patients, primarily treated by (chemo)radiation was performed. In total 42 studies, concerning 82 cell biological markers were included. Only a small number of markers were investigated in more than one independent study, therefore cell biological markers were clustered on biological function. Clusters with the most potential prognostic factors were markers involved in angiogenesis and hypoxia, mainly immunostaining of CA9 and HIF-1α, and markers involved in the EGFR pathway, especially protein expression of EGFR and C-erbB-2. Furthermore, COX-2 immunostaining and serum SCC-ag levels appear to be prognostic markers. Positivity of all these cell biological markers was associated with poor survival. For most markers the association with poor prognosis was found in both univariate and multivariate analysis, indicating that these cell biological markers give additional information on prognosis, complementary to clinicopathological prognostic factors. The relation with response to (chemo)radiation was determined in 38/82 markers. EGFR, C-erbB-2, and COX-2 were also associated with poor response to the-

rapy, while markers involved in angiogenesis and hypoxia did not show a relation with response. Serum SCC-ag levels were not investigated in relation to response. Besides the individual prognostic significance of these markers, it is even more interesting that also relations exist between COX-2, the EGFR pathway and angiogenesis and hypoxia. Forexample, COX-2 derived prostaglandin E_2 (PGE_2) activates the EGFR pathway, which in turn results in increased COX-2 expression (7). Furthermore, HIF-1α can induce expression of COX-2 under hypoxic conditions and the elevated levels of PGE_2 promote transcriptional activity of HIF-1α and expression of VEGF, also indicating a positive feedback mechanism (8). Targeted treatments against these cell biological markers are already under development.

The prognostic value of different components of the EGFR pathway in cervical cancer is equivocal, which is due to small, heterogeneous (frequently a mix of primarily surgically and radiotherapeutically treated) patient populations. The aim of **chapter 3** was to determine the relation between proteins involved in the EGFR pathway and survival in a well-documented series of early stage (Ib-IIa) cervical cancer patients primarily treated by radical hysterectomy and pelvic lymph node dissection. Immunostaining for EGFR, phosphorylated (p)EGFR, PTEN, pAKT, and pERK was performed on tissue microarrays (TMAs) containing 336 consecutive early stage cervical cancer patients and related to clinicopathological characteristics and disease-specific survival. Positive PTEN immunostaining was associated with absence of pelvic lymph node metastasis (OR=0.51; 95%CI=0.30 – 0.90; *P*=0.019). This suggests that PTEN is one of the tumor suppressor genes affecting pelvic lymph node metastasis. However, expression of the various EGFR pathway components does not appear to have prognostic impact in surgically treated early stage cervical cancer.

The aim of **chapter 4** was to identify cellular tumor pathways associated with pelvic lymph node metastasis in early stage cervical cancer. To identify such pathways, expression array analysis (Affymetrix U133 plus 2.0 microarrays) was performed of tumor tissues of 20 patients with histologically confirmed lymph node metastases vs. 19 patients with histologically and clinically confirmed negative lymph nodes. Gene Set Enrichment Analysis (GSEA) (9) and a method in which experimentally generated expression signatures that reflect the activation of various oncogenic signaling pathways are used (10), revealed that the TGF-β pathway was significantly enriched in the group of patients with negative lymph nodes (*P*=0.027), while dysregulation of the β-catenin pathway was associated with presence of pelvic lymph node metastases (*P*=0.001). In addition, individual genes that were differentially expressed were identified (*P*<0.001) and within the group of 149 differentially expressed genes (188 probe sets), several were

involved in the β-catenin pathway (*TCF4*, *CTNNAL1*, *CTNND1/p120*, *DKK3* and *WNT5a*). As validation, immunostaining of TMAs containing 274 consecutive early stage cervical cancer patients, primarily treated with surgery, was performed for Smad4 (TGF-β pathway), and β-catenin and p120 (β-catenin pathway). This revealed that indeed positivity of Smad4 was related to absence of lymph node metastases (OR=0.20; 95%CI=0.06 – 0.66), while p120 positivity was related to positive lymph nodes (OR=1.79; 95%CI=1.05 – 3.05). β-catenin itself, however, was not related to lymph node metastases. In conclusion, this study provides new, validated, insights in the molecular mechanism of lymph node metastases in cervical cancer. The TGF-β pathway is associated with negative lymph nodes, while the p120-associated β-catenin pathway is predictive of pelvic lymph node metastases. Ultimately, more detailed analysis of these pathways might result in the identification of additional markers that will increase the clinical sensitivity and specificity for prediction of pelvic lymph node metastases in cervical cancer.

In **chapter 5** protein expression of DR4, DR5, and TRAIL in cervical cancer was studied and their prognostic and predictive value was determined. For this purpose, immunostaining of DR4, DR5, and TRAIL was performed on TMAs containing 645 cervical cancer patients primarily treated by radical surgery and/or (chemo)radiation. Immunostaining was related to response to (chemo)radiation (defined as residual tumor tissue in a biopsy 8-10 weeks after completion of treatment) and disease-specific survival. Weak cytoplasmatic DR4, DR5, and TRAIL immunostaining were observed in cervical tumors from 99%, 88%, and 81% of the patients, respectively. No membranous staining was observed. In patients treated primarily with (chemo)radiation, positivity of TRAIL was associated with residual tumor tissue after completion of treatment (OR=2.09; 95%CI=1.01 – 4.33; P<0.05). However, positive DR4, DR5, and TRAIL expression were not prognostic for disease-specific survival. In conclusion, DR4, DR5 and TRAIL are frequently expressed in cervical cancer, while immunostaining of DR4, DR5, and TRAIL do not show prognostic significance.

Besides assessing the prognostic role of EGFR and its pathways members in early stage cervical cancer patients primarily treated by surgery, in **chapter 6** the EGFR pathway was studied in locally advanced stage cervical cancer patients primarily treated by (chemo)radiation. Immunostaining was performed for EGFR, pEGFR, PTEN, pAKT, and pERK, on TMAs that contain 375 consecutive FIGO stage Ib-IVa cervical cancer patients, primarily treated with (chemo)radiation. Immunostaining was related to response to treatment and survival. Response to treatment was assessed in two models. In model I response to (chemo)radiation was determined by locoregional

disease-free survival in all patients. In order to be able to analyze two populations with supposedly the highest difference in sensitivity to (chemo)radiation, in model II two populations with optimal and very poor response to (chemo)radiation were defined. Membranous staining of EGFR (HR=1.84; 95%CI=1.20 – 2.82; *P*=0.005) and cytoplasmic staining of pEGFR (HR=1.71; 95%CI=1.11 – 2.66; *P*=0.016) were both independent predictors of poor response to (chemo)radiation (model I). The relation of both EGFR (OR=6.08; 95%CI=2.39 – 15.47; *P*<0.001) and pEGFR (OR=4.06; 95%CI=1.58 – 10.43; *P*=0.004) with response was the strongest in our model with the highest contrast in radiosensitivity (model II), supporting the idea that indeed EGFR and pEGFR are associated with a poor response to (chemo)radiation in cervical cancer. Moreover, membranous EGFR staining was an independent prognostic factor for poor disease-specific survival (HR=1.54; 95%CI=1.09 – 2.17; *P*=0.014). In advanced stage cervical cancer patients, the apparent involvement of EGFR in response to (chemo)radiation presents the EGFR pathway as a promising therapeutic target in already ongoing clinical trials, in which EGFR inhibitors are combined with standard chemoradiation in cervical cancer patients.

Finally, the aim of **chapter 7** was to examine the role of protein expression of non-phosphorylated ATM (nATM) and phosphorylated ATM (pATM) in response to (chemo)radiation and disease-specific survival in 375 consecutive FIGO stage Ib-IVa cervical cancer patients, primarily treated with (chemo)radiation. Protein expression of nATM and pATM was assessed by immunohistochemistry on TMAs. Again the models of chapter 6 were used to determine the relation with response to treatment (model I: locoregional disease-free survival in all patients; model II: two populations with optimal and very poor response to (chemo)radiation). In univariate analysis, high nATM (HR=1.83; 95%CI=1.14 – 2.96; *P*=0.013) as well as high pATM (HR=1.87; 95%CI=1.22 – 2.86; *P*=0.004) staining were associated with poor response to

(chemo)radiation in model I. The association was again the strongest in model II, with the highest contrast in radiosensitivity (nATM: OR=3.08; 95%CI=1.32 – 7.18; *P*=0.009; pATM: OR=2.60; 95%CI=1.26 – 5.37; *P*=0.010). In multivariate analysis, high pATM was a predictor of poor response, independent of well-known clinicopathological prognostic factors (Model I: HR=1.64; 95%CI=1.02 – 2.66; *P*=0.043; Model II: OR=2.36; 95%CI=1.05 – 5.30; *P*=0.038). Furthermore, high pATM immunostaining was related to poor disease-specific survival (HR=1.49; 95%CI=1.06 – 2.08; *P*=0.020). These results present ATM as an interesting therapeutic target in cervical cancer.

Conclusions and future perspectives

Over the last decades, large numbers of cell biological markers have been studied to get more insight in the cell biology of cervical cancer and to identify potential targets for therapeutic intervention (6). For example, over-expression of EGFR has frequently been observed in human cancers and often associated with poor outcome after curative radiotherapy, for instance in head and neck squamous cell carcinoma (11,12). This might be explained by the fact that EGFR is involved in DNA double strand break (DSB) repair after radiotherapy (13). Because of the apparent involvement of EGFR in response to (chemo)radiation, EGFR targeted therapy has recently been implemented as a new therapeutic strategy in various malignancies (14). The results of chapter 6 show that EGFR immunostaining as well as pEGFR immunostaining were also predictive of poor response to (chemo)radiation in cervical cancer patients, indicating that locally advanced stage cervical cancer patients might benefit from EGFR targeting treatment in combination with standard chemoradiation. At the moment, clinical trials are ongoing, in which cetuximab, a chimeric human mouse anti-EGFR monoclonal antibody, is added to standard chemoradiation (http://www.cancer.gov/clinicaltrials, NCT00104910, NCT00292955, NCT00957411).

Comparable data have been presented for ATM. Preclinical studies on the role of ATM in response to radiotherapy, showed that downregulation of ATM, resulted in the radiosensitization of cervical cancer cells (15,16). The role of ATM in response to radiotherapy is probably due to its key role in DNA DSB signaling. Upon DSB DNA damage ATM is activated through phosphorylation, which results in phosphorylation of target proteins resulting in DNA repair (17). Chapter 7 indicates that indeed immunostaining of nATM and pATM are associated with poor response to (chemo)radiation in locally advanced stage cervical cancer. These results present ATM as an interesting therapeutic target. However, nowadays no FDA approved ATM inhibitor is available and therefore, this should be the focus of future research.

Besides enhancing response to (chemo)radiation, targeted drugs may also inhibit metastatic potential of tumor cells. Angiogenesis and hypoxia are essential for growth and progression of cancer and a relation with metastases has also been suggested (18,19). As established in the systematic review (chapter 2), expression of multiple proteins involved in angiogenesis and hypoxia was associated with poor (metastases-free) survival. Inhibition of angiogenesis is nowadays of major interest and is currently tested in metastatic and recurrent cervical cancer (http://www.cancer.gov/clinicaltrials, NCT00803062). In chapter 4 the TGF-β and the p120-associated β-catenin pathway were identified to be important in cervical cancer pelvic lymph node metastases.

Targeted therapies have been reported for both pathways (20,21). Although both pathways are known for effecting metastatic potential, more research is needed to elucidate whether targeting these pathways will inhibit metastatic potential of tumor cells *in vitro* and *in vivo*.

A major challenge for the efficient implementation of new targeted treatment strategies is to select those patients, who will really benefit, thereby avoiding overtreatment and unnecessary side-effects. Predictive cell biological markers can be helpful in selecting patients who are likely to have a good response to the targeted treatment. For instance, in colorectal cancer and non small cell lung cancer *KRAS* mutations are associated with resistance to EGFR inhibitors (22). Specific *EGFR* mutations and high copy numbers of the *EGFR* gene predict a better response in non-small cell lung cancer (23,24), while protein expression levels were not predictive of response to cetuximab in colorectal cancer patients (25,26). In cervical cancer neither *EGFR* mutations (27), nor *EGFR* gene amplification (28), and only a few (0-8%) *KRAS* mutations (29-32) have been observed. Therefore, these markers will probably not be helpful in predicting response to EGFR targeted therapy in cervical cancer. If EGFR targeted treatment in addition to standard chemoradiation will result in a survival benefit in cervical cancer, additional research should be performed to identify cell biological markers that can predict which patients will actually benefit from this treatment.

Besides selection of patients who are likely to benefit from targeted treatments, cell biological markers may also allow better prediction of likelihood of poor prognostic factors, such as pelvic lymph node metastases. If presence of lymph nodes metastases is known prior to treatment, primary chemoradiation can be considered instead of surgery in combination with (chemo)radiation, which is equally effective, but associated with a different treatment associated morbidity pattern (2). Although the number of reports on prognostic markers in cervical cancer is rising per month, their application in clinical practice nowadays is limited. The choice of treatment is still mainly based on clinicopathological characteristics such as FIGO stage, due to the fact that currently in cervical cancer no molecular diagnostic marker exists that has been validated such that implementation in clinical practice is indicated. Reasons for slow implementation of this type of markers in cervical cancer are: 1) the predictive power eg. for presence of pelvic lymph node metastases needs to be very strong in order to allow treatment choices, 2) cell biological markers are often studied in small, heterogeneous patient populations that are retrospectively selected, 3) differences in methodology exist between studies reporting on predictive markers, for example, different antibodies are used for immunohistochemical staining and, more important, most studies do not use similar

cut-offs score with a certain rationale for positivity of the marker. As a consequence, results are often not reproducible. In order to improve quality of studies reporting on prognostic markers, McShane *et al.* proposed the REMARK (REporting recommendations for tumour MARKer prognostic studies) criteria (33). Improving quality of studies reporting prognostic markers, should lead to more reliable conclusions about the prognostic significance of cell biological markers and finally to application in clinical decision-making.

As became clear from our systematic review (chapter 2), the majority of studies reporting on prognostic and predictive markers in cervical cancer, only investigated one or two related cell biological markers in small and often heterogeneous patient populations. As a result, often different conclusions can be drawn from studies reporting on the same cell biological marker. The use of the TMA technology, as applied in various chapters of this thesis, allows rapid immunohistochemical staining and analysis of many tissue specimens in one experiment, while saving precious tumor tissue (34). Therefore, we were able to evaluate several proteins of one pathway in large consecutive series of cervical cancer patients. However, nowadays the focus of identification of new prognostic and predictive markers lies more on high-throughput microarray technology, in which gene expression levels of enormous numbers of genes can be determined in only one experiment and patterns of biological differences between cancers can be identified (35,36). In this way, single genes as well as pathways that are associated with lymph node metastases (as shown in chapter 4), response to treatment and prognosis can be discovered. This approach might contribute to individual therapeutic strategies in cervical cancer (37).

In conclusion, this thesis presents new insights in the molecular mechanism of lymph node metastases, response to (chemo)radiation and prognosis and in cervical cancer. Identification of cell biological markers predictive for response to treatment may identify new targets for therapeutic intervention. Cell biological markers may also contribute to appropriate selection of patients who are likely to benefit from targeted therapies. Ultimately, this will support optimization of cervical cancer treatment and thereby improve overall survival rates.

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Summary in Dutch

Nederlandse samenvatting

Samenvatting

Baarmoederhalskanker is na borst- en darmkanker de meest voorkomende vorm van kanker bij vrouwen wereldwijd. In 2008 werden 529.000 nieuwe patiënten gediagnosticeerd en stierven 275.000 patiënten aan deze vorm van kanker (1). In ontwikkelde landen heeft de invoering van nationale screeningsprogramma's op (voorstadia van) baarmoederhalskanker gezorgd voor een sterke daling van het aantal nieuwe patiënten met baarmoederhalskanker (2). Echter in ontwikkelingslanden vertegenwoordigt baarmoederhalskanker nog steeds een groot deel van de maligniteiten bij vrouwen (1). Een infectie met het hoog-risico humaan papillomavirus (hr-HPV) is de belangrijkste oorzaak van het ontstaan van baarmoederhalskanker (3). Daarom is men tegenwoordig gericht op het verbeteren van de screening op baarmoederhalskanker, middels het testen op aanwezigheid van hr-HPV DNA en zijn er vaccins tegen HPV ontwikkeld (4,5).

De behandeling van vroeg stadium baarmoederhalskanker (stadium Ia-IIa) bestaat uit een radicale uterusextirpatie en pelviene lymfklierdissectie. In deze groep patiënten is de aanwezigheid van pelviene lymfkliermetastasering de belangrijkste prognostische factor (6). Patiënten met vroeg stadium baarmoederhalskanker zonder lymfkliermetastasen hebben een 5-jaarsoverleving van ongeveer 90%, tegenover 65% bij patiënten met lymfkliermetastasen (7). Daarom worden deze patiënten aanvullend behandeld met radiotherapie of chemoradiotherapie. De combinatie van chirurgische behandeling gevolgd door (chemo)radiotherapie is echter geassocieerd met ernstige morbiditeit (8). Wanneer de aanwezigheid van lymfkliermetastasen bekend zou zijn voor de start van de behandeling, zou primaire behandeling met chemoradiotherapie overwogen kunnen worden. Dit is even effectief als primair chirurgische behandeling, gevolgd door chemoradiotherapie, maar geassocieerd met minder morbiditeit (8). Op dit moment zijn er nog geen klinisch-pathologische factoren of celbiologische markers beschikbaar die aanwezigheid van lymfkliermetastasen met hoge sensitiviteit en specificiteit kunnen voorspellen. De toepassing van weinig of niet invasieve technieken, zoals de schildwachtklierprocedure, wordt momenteel onderzocht om patiënten met lymfkliermetastasen beter te kunnen opsporen (9).

De behandeling van patiënten met lokaal gevorderde baarmoederhalskanker (stadium Ib2, IIb-IVa) bestaat uit gecombineerde radio- en chemotherapie (cisplatinum), omdat dit effectiever bleek te zijn dan radiotherapie alleen (10). De toepassing van chemoradiotherapie heeft de overleving verbeterd en het ontstaan van zowel lokale recidieven als afstandsmetastasen verminderd (11-13). De respons op radiotherapie kan beoordeeld worden door gynaecologisch onderzoek met het nemen van biopten onder algehele narcose, 8 tot 10 weken na het beëindigen van de primaire behandeling. Als

hierbij nog vitaal tumorweefsel in biopten wordt gevonden kan een adjuvante (radicale) uterusextirpatie worden uitgevoerd (14). Ondanks dat de invoering van chemoradiotherapie de overlevingskans heeft verbeterd, is de 5-jaars overleving van patiënten met lokaal gevorderde ziekte slechts 66% (10). Verbetering van de overleving door de standaard behandeling te intensiveren is niet goed mogelijk, vanwege resistentie tegen chemo- en/of radiotherapie en een toename van bijwerkingen op korte en lange termijn (15). Daarom zijn dringend nieuwe behandelingsmodaliteiten nodig, die de effectiviteit van chemoradiotherapie verhogen om zo de overleving in patiënten met lokaal gevorderde baarmoederhalskanker te verbeteren.

De keuze voor behandeling is op dit moment vooral gebaseerd op bekende klinisch-pathologische factoren, zoals stadium en aanwezigheid van lymfkliermetastasen. Daarnaast zouden ook celbiologische factoren hierin een rol kunnen spelen. Het aantonen van relaties van celbiologische markers met respons op chemoradiotherapie en/of prognose dient te leiden tot de identificatie van nieuwe moleculaire aangrijpingspunten voor therapeutische interventie. De combinatie van zogenaamde doelgerichte of targeted therapieën met standaard chemoradiotherapie zou idealiter moeten resulteren in een verbetering van de overleving, zonder verhoging van de toxiciteit (16).

In dit proefschrift is de relatie tussen celbiologische markers, intracellulaire routes en lymfkliermetastasering, respons op (chemo)radiotherapie en prognose in baarmoederhalskanker onderzocht.

In **hoofdstuk 2** is een systematische review uitgevoerd van goed gedocumenteerde studies over de prognostische en predictieve (voorspellende) waarde van celbiologische markers in baarmoederhalskanker, primair behandeld met (chemo)radiotherapie. In totaal zijn 42 studies, betreffende 82 celbiologische markers in het systematische review geïncludeerd. Slechts een klein aantal markers is bestudeerd in meer dan één onafhankelijk onderzoek en daarom zijn de celbiologische markers geclusterd op basis van hun biologische functie. Clusters met de meest veel belovende prognostische markers, bleken markers te bevatten, die betrokken zijn bij angiogenese en hypoxie, met name eiwitexpressie van CA9 en HIF-1α en tevens markers die een rol spelen in de EGFR pathway, met name eiwitexpressie van EGFR and C-erbB-2. Verder zijn eiwitexpressie van COX-2 en serum SCC-ag van prognostische waarde. Sterkere expressie was voor al deze markers gerelateerd aan een slechtere prognose. De relatie met prognose is voor de meeste markers zowel gevonden in univariate als in multivariate analyse, hetgeen aangeeft dat deze markers toegevoegde prognostische waarde hebben, bovenop de al bekende klinisch-pathologische prognostische factoren. Naast de prognostische waarde, is ook de relatie met respons op (chemo)radiotherapie onderzocht voor 38/82 markers.

Hieruit bleek dat EGFR, C-erbB-2 en COX-2 ook geassocieerd zijn met een slechte respons op behandeling. Dit werd echter niet gevonden voor markers die betrokken zijn bij angiogenese en hypoxie. De waarde van de bevindingen van dit systematische review wordt onderstreept door het feit dat er naast de individuele prognostische waarde, ook onderlinge relaties bestaan tussen COX-2, de EGFR pathway en angiogenese en hypoxie. Een voorbeeld hiervan is dat prostaglandine E_2 (PGE_2), dat door COX-2 tot expressie wordt gebracht, leidt tot activatie van de EGFR pathway. Dit resulteert vervolgens weer in verhoogde expressie van COX-2 (17). Ook is er een positief feedback mechanisme beschreven voor HIF-1α en COX-2; HIF-1α zorgt voor verhoogde COX-2 expressie onder hypoxische omstandigheden, waarna verhoogde expressie van PGE_2 leidt tot een toename van de HIF-1α activiteit en VEGF expressie (18). Doelgerichte behandelingen voor al deze markers worden momenteel ontwikkeld.

Momenteel is de prognostische waarde van verschillende eiwitten uit de EGFR pathway in patiënten met vroeg stadium baarmoederhalskanker nog onduidelijk. Dit komt omdat deze eiwitten tot nu toe onderzocht zijn in kleine en diverse (vaak zowel primair chirurgisch als radiotherapeutisch behandelde) patiëntenpopulaties. Het doel van **hoofdstuk 3** was het bepalen van de relatie tussen eiwitten die betrokken zijn bij de EGFR pathway en overleving in een grote, goed gedocumenteerde populatie vroeg stadium baarmoederhalskanker patiënten die primair behandeld zijn met radicale uterusextirpatie en pelviene lymfklierdissectie. Hiervoor is immunohistochemie toegepast voor EGFR, gefosforyleerd (p)EGFR, PTEN, pAKT en pERK, op tissue microarrays (TMAs) die tumorweefsel bevatten van 336 vroeg stadium baarmoederhalskanker patiënten. Eiwitexpressie werd gerelateerd aan klinisch-pathologische factoren en ziektespecifieke overleving. Positieve PTEN expressie was gerelateerd aan afwezigheid van lymfkliermetastasen. Dit impliceert dat PTEN mogelijk één van de tumor suppressor genen is betrokken bij lymfkliermetastasering. Verder had eiwitexpressie van de diverse componenten van de EGFR pathway geen prognostische waarde bij chirurgisch behandelde vroeg stadium baarmoederhalskanker patiënten.

In **hoofdstuk 4** zijn intracellulaire routes (pathways) die geassocieerd zijn met lymfkliermetastasering in vroeg stadium baarmoederhalskanker onderzocht. Voor identificatie van dergelijke pathways is genexpressie microarray analyse (Affymetrix U133 plus 2.0 microarrays) gedaan op tumorweefsel van 20 patiënten met histologisch bevestigde lymfkliermetastasen en van 19 patiënten zonder lymfkliermetastasen, zowel histologisch als klinisch bevestigd. Gene Set Enrichment Analysis (GSEA) (19) en een methode waarin experimenteel gegenereerde expressie profielen worden gebruikt die activatie van verschillende oncogene pathways weerspiegelen (20), zijn toegepast om

pathways te identificeren die geassocieerd zijn met lymfkliermetastasering. Slechts een paar functionele groepen van genen bleken een relatie te hebben met lymfkliermetastasering, waaronder twee bekende pathways. De TGF-β pathway was significant geassocieerd met afwezigheid van lymfkliermetastasen. Daarentegen was ontregeling van de β-catenin pathway geassocieerd met aanwezigheid van lymfkliermetastasen. Verder zijn individuele genen geïdentificeerd die verschillend tot expressie kwamen in patiënten met en zonder lymfkliermetastasen. Binnen de groep van 149 genen (188 probe sets) waren verschillende genen betrokken bij de β-catenin pathway (*TCF4*, *CTNNAL1*, *CTNND1/p120*, *DKK3* en *WNT5a*). Om deze resultaten te valideren is immunohistochemie verricht voor Smad4 (TGF-β pathway), en β-catenin en p120 (β-catenin pathway) op TMAs die weefsel bevatten van 274 vroeg stadium baarmoederhalskanker patiënten, die primair chirurgisch behandeld zijn. In overeenstemming met de geïdentificeerde pathways, bleek positieve expressie van Smad4 gerelateerd te zijn aan afwezigheid van lymfkliermetastasering en p120 was geassocieerd met aanwezigheid van lymfkliermetastasen, terwijl β-catenin zelf echter niet geassocieerd was met lymfkliermetastasen. Concluderend geeft deze studie nieuwe, extern gevalideerde, inzichten in moleculaire mechanismen van lymfkliermetastasering in baarmoederhalskanker. De TGF-β pathway is geassocieerd met afwezigheid van lymfkliermetastasen, terwijl de p120-geassocieerde β-catenin pathway voorspellend is voor aanwezigheid van lymfkliermetastasen. Uiteindelijk zou gedetailleerde kennis van deze pathways kunnen leiden tot identificatie van meer markers die met hogere sensitiviteit en specificiteit lymfkliermetastasen kunnen voorspellen in baarmoederhalskanker patiënten.

In **hoofdstuk 5** is de predictieve en prognostische waarde van eiwitexpressie van DR4, DR5 en TRAIL in baarmoederhalskanker onderzocht. Hiervoor werd eiwitexpressie van DR4, DR5 en TRAIL bepaald door middel van immunohistochemie op TMAs die weefsel bevatten van 645 baarmoederhalskanker patiënten die primair behandeld zijn met chirurgie en/of (chemo)radiotherapie. Eiwitexpressie werd gerelateerd aan respons op (chemo)radiotherapie (gedefinieerd als aanwezigheid van vitaal tumor weefsel in een biopt dat genomen is 8-10 weken na het beëindigen van de behandeling) en aan ziektespecifieke overleving. Zwak cytoplasmatische aankleuring van DR4, DR5 en TRAIL werd waargenomen in respectievelijk 99%, 88% en 81% van de tumoren. In de patiënten primair behandeld met (chemo)radiotherapie was positieve expressie van TRAIL gerelateerd aan aanwezigheid van vitaal tumor weefsel 8-10 weken na het beëindigen van de behandeling. DR4, DR5 en TRAIL bleken echter geen prognostische factoren voor ziektespecifieke overleving te zijn. Concluderend komen DR4, DR5 en TRAIL vaak tot expressie in baarmoederhalskanker, maar hebben ze geen prognostische waarde.

Naast het onderzoek in vroeg stadium baarmoederhalskanker patiënten die primair chirurgisch behandeld zijn naar de predictieve en prognostische waarde van EGFR en andere eiwitten betrokken bij deze pathway, is in **hoofdstuk 6** dezelfde EGFR pathway bestudeerd in patiënten met lokaal gevorderde baarmoederhalskanker, die primair behandeld zijn met (chemo)radiotherapie. Immunohistochemie werd toegepast voor EGFR, pEGFR, PTEN, pAKT en pERK, op TMAs die tumorweefsel bevatten van 375 stadium Ib-IVa baarmoederhalskanker patiënten. Immunohistochemische aankleuring werd gerelateerd aan de respons op behandeling en overleving. Respons op behandeling werd bepaald in twee verschillende modellen. In model I is de respons op (chemo)radiotherapie bepaald op basis van de locoregionale ziektevrije overleving in alle patiënten. In model II zijn twee groepen gedefinieerd; één groep met een hele goede respons en één met een hele slechte respons op (chemo)radiotherapie, om zo de twee meest extreme populaties met elkaar te kunnen vergelijken. Aanwezigheid van EGFR op de celmembraan (en aanwezigheid van pEGFR in het cytoplasma bleken allebei voorspellend te zijn voor een slechte respons op (chemo)radiotherapie (model I). De relatie van zowel EGFR als pEGFR met respons was het sterkst in model II, hetgeen erop wijst dat er daadwerkelijk een relatie bestaat tussen EGFR en pEGFR met respons op (chemo)radiotherapie in baarmoederhalskanker. Verder was membraan aankleuring van EGFR ook een onafhankelijke prognostische factor voor slechte ziektespecifieke overleving. De rol die EGFR speelt in de respons op (chemo)radiotherapie, presenteert de EGFR pathway als een veelbelovend therapeutisch aangrijpingspunt in klinische studies die momenteel uitgevoerd worden, waarin anti-EGFR therapie gecombineerd wordt met standaard chemoradiotherapie in baarmoederhalskanker patiënten.

Tot slot is in **hoofdstuk 7** de rol van niet-gefosforyleerd ATM (nATM) en gefosforyleerd ATM (pATM) bepaald in relatie tot respons op (chemo)radiotherapie en ziektespecifieke overleving in 375 stadium Ib-IVa baarmoederhalskanker patiënten, die primair behandeld zijn met (chemo)radiotherapie. Eiwitexpressie werd bepaald door middel van immunohistochemie op TMAs. Om de respons op (chemo)radiotherapie te bepalen werd opnieuw gebruik gemaakt van de modellen uit hoofdstuk 6 (model I: locoregionale ziektevrije overleving in alle patiënten; model II: twee groepen patiënten met goede en slechte respons op (chemo)radiotherapie). In univariate analyse was zowel hoge nATM expressie als hoge pATM expressie gerelateerd aan slechte respons in model I. Opnieuw was de relatie het sterkst in model II, met het grootste verschil in radiosensitiviteit. In de multivariate analyse was hoge pATM expressie een voorspeller van slechte respons op (chemo)radiotherapie, onafhankelijk van de bekende klinisch-pathologische prognostische factoren. Ook was hoge pATM expressie geassocieerd met een slechte

ziektespecifieke overleving. Concluderend blijkt uit deze studie dat ATM een potentieel therapeutisch aangerijpingspunt is in de behandeling van baarmoederhalskanker.

Conclusies en toekomstvisie

Meer kennis over welke celbiologische processen een rol spelen in het biologische gedrag van baarmoederhalskanker dient te leiden tot toepassing van deze kennis bij de diagnostiek en behandeling van patiënten met baarmoederhalskanker. Een voorbeeld hiervan is dat EGFR over-expressie niet alleen frequent wordt gevonden in baarmoederhalskanker, maar ook in andere maligniteiten, zoals hoofd-halskanker en dat dit ook hierin vaak geassocieerd is met slechte uitkomst van radiotherapeutische behandeling (21,22). Dit kan verklaard worden doordat EGFR betrokken is bij herstel van DNA schade na radiotherapie (23). Vanwege de rol die EGFR speelt in de respons op (chemo)radiotherapie, is anti-EGFR behandeling de afgelopen tijd geïmplementeerd als nieuwe therapeutische strategie in verschillende soorten kanker (24). De resultaten van hoofdstuk 6 laten zien dat zowel EGFR als pEGFR eiwitexpressie slechte respons op (chemo)radiotherapie in baarmoederhalskanker voorspellen. Dit suggereert dat ook patiënten met lokaal gevorderde baarmoederhalskanker baat zouden kunnen hebben bij anti-EGFR behandeling in combinatie met standaard (chemo)radiotherapie. Op dit moment zijn klinische studies gaande, waarin cetuximab, een chimerisch monoklonaal antilichaam gericht tegen EGFR, wordt gecombineerd met chemoradiotherapie (http://www.cancer.gov/clinicaltrials, NCT00104910, NCT00292955, NCT00957411).

Vergelijkbare resultaten zijn gepubliceerd voor ATM. Preklinische studies naar de rol van ATM in de respons op (chemo)radiotherapie hebben laten zien dat afname van ATM een verhoogde radiosensitiviteit in baarmoederhalskanker cellijnen tot gevolg had (25,26). De rol van ATM in de respons op radiotherapie wordt waarschijnlijk veroorzaakt door de belangrijke rol die ATM speelt in herstel van DNA schade. Na het ontstaan van DNA schade wordt ATM geactiveerd door fosforylatie. Dit resulteert in fosforylatie van downstream genen die betrokken zijn bij herstel van DNA schade (27). Hoofdstuk 7 laat zien dat eiwitexpressie van nATM en pATM inderdaad gerelateerd is aan een slechte respons op (chemo)radiotherapie in laat stadium baarmoederhalskanker patiënten. Gebaseerd op deze resultaten kan ATM dus gezien worden als een potentieel therapeutisch aangrijpingspunt. Op dit moment is echter geen goedgekeurde anti-ATM behandeling beschikbaar, dus hier zal in de toekomst verder onderzoek naar gedaan moeten worden.

Naast doelgerichte behandelingen om de respons op (chemo)radiotherapie te verbeteren, zouden deze ook gebruikt kunnen worden om het ontstaan van metastasen te voorkomen. Angiogenese en hypoxie zijn essentieel voor groei en progressie van kanker en ook is er een relatie met metastasering beschreven (28,29). Zoals duidelijk is geworden uit het systematische review (hoofdstuk 2) is expressie van verschillende eiwitten die betrokken zijn bij angiogenese en hypoxie geassocieerd met slechtere (metastasevrije) overleving. Daarom wordt tegenwoordig veel aandacht geschonken aan inhibitie van angiogenese en wordt dit momenteel onderzocht in patiënten met gerecidiveerde en/of gemetastaseerde baarmoederhalskanker (http://www.cancer.gov/clinicaltrials, NCT00803062). Uit hoofdstuk 4 is gebleken dat de TGF-β en de p120-geassocieerde β-catenin pathway belangrijke pathways lijken te zijn voor lymfklier metastasering in baarmoederhalskanker. Er zijn doelgerichte behandelingen beschreven voor beide pathways (30,31), daarom zou verder onderzocht moeten worden of deze behandelingen kunnen leiden tot preventie van metastasering in baarmoederhalskanker.

Een grote uitdaging voor efficiënte implementatie van nieuwe doelgerichte behandelingsstrategieën is om voor de behandeling alleen de patiënten te selecteren die er ook daadwerkelijk baat bij zullen hebben. Op deze manier zouden overbehandeling en daarmee onnodige bijwerkingen voorkomen kunnen worden. Predictieve celbiologische markers dienen bij te dragen aan selectie van patiënten die een goede respons op de doelgerichte therapie zullen hebben. In darmkanker en niet-kleincellige longkanker zijn mutaties in het *KRAS* gen bijvoorbeeld geassocieerd met resistentie tegen anti-EGFR therapie (32). Verder voorspellen specifieke *EGFR* mutaties en amplificatie van het *EGFR* gen een betere respons op anti-EGFR behandelingen in longkanker (33,34). Daarentegen is het eiwitexpressie niveau niet voorspellend voor respons op cetuximab in darmkanker patiënten (35,36). In baarmoederhalskanker worden echter geen *EGFR* mutaties (37), of *EGFR* gen amplificatie gevonden (38) en slechts weinig *KRAS* mutaties (0-8%) (39-42). Daarom zullen deze markers waarschijnlijk niet kunnen bijdragen aan het voorspellen van de respons op anti-EGFR behandelingen in baarmoederhalskanker. Als inderdaad blijkt dat anti-EGFR behandeling in combinatie met chemoradiotherapie een gunstig effect heeft op de overleving zal verder onderzoek gedaan moeten worden naar celbiologische markers die kunnen voorspellen welke patiënten met name baat zullen hebben bij deze behandeling.

Naast het selecteren van patiënten voor doelgerichte therapieën, zouden prognostische celbiologische markers een rol kunnen spelen in het voorspellen van aanwezigheid van ongunstige prognostische factoren, zoals aanwezigheid van lymfkliermetastasen. Als aanwezigheid van lymfkliermetastasen adequaat voorspeld zou kunnen worden vooraf-

gaand aan de behandeling, zou primaire chemoradiotherapie overwogen kunnen worden in plaats van chirurgische behandeling in combinatie met (chemo)radiotherapie. Dit is namelijk net zo effectief gebleken, maar het is geassocieerd met minder morbiditeit (8). Ondanks dat het aantal studies over predictieve en prognostische markers in baarmoederhalskanker per maand stijgt, worden ze op dit moment niet of nauwelijks gebruikt in de dagelijkse praktijk. Oorzaken hiervoor zijn: 1) de voorspellende waarde voor bijvoorbeeld aanwezigheid van lymfkliermetastasen moet zeer sterk zijn om de keuze voor een behandeling er vanaf te laten hangen; 2) cel biologische markers worden vaak slechts bestudeerd in kleine, heterogene en retrospectief verzamelde patiënten populaties; 3) er bestaan veel methodologische verschillen tussen studies. Zo worden er bijvoorbeeld verschillende antilichamen gebruikt voor immunohistochemische kleuringen en nog belangrijker is dat er verschillende afkapwaarden voor positiviteit van een marker worden gebruikt, zonder dat daar een reden voor wordt gegeven. Het gevolg hiervan is dat resultaten vaak niet reproduceerbaar zijn. Om de kwaliteit van studies over prognostische markers te verbeteren hebben McShane *et al.* de REMARK (REporting recommendations for tumour MARKer prognostic studies) criteria opgesteld (43). Een verbetering van de kwaliteit van studies naar prognostische markers dient te leiden tot meer betrouwbare conclusies over de prognostische waarde van celbiologische markers en uiteindelijk tot toepassing van deze markers in klinische besluitvorming.

Zoals duidelijk is geworden uit het systematische review (hoofdstuk 2), bestuderen de meeste studies naar predictieve en prognostische markers slechts één of twee aan elkaar gerelateerde celbiologische markers in vaak kleine, gevarieerde patiënten populaties. Het gevolg hiervan is dat regelmatig verschillende conclusies getrokken kunnen worden uit studies die onderzoek doen naar dezelfde celbiologische marker. Het gebruik van TMAs, zoals toegepast in diverse hoofdstukken van dit proefschrift, maakt snelle immunohistochemische kleuring en analyse mogelijk van grote patiënten aantallen in slechts één experiment, terwijl kostbaar tumor weefsel gespaard wordt (44). Dit maakt het mogelijk dat wij verschillende eiwitten uit één pathway konden bestuderen in grote groepen baarmoederhalskanker patiënten.

Een relatief nieuwe methode voor identificatie van nieuwe predictieve en prognostische markers is genexpressie microarray technologie, waarmee genexpressie niveaus van in principe alle genen tegelijkertijd kunnen worden bestudeerd (45,46). Op deze manier kunnen aanwijzingen voor zowel individuele genen als intracellulaire tumor routes die betrokken zijn bij lymfkliermetastasering (hoofdstuk 4), respons op therapie en prognose worden verkregen, die vervolgens in nadere studies gevalideerd dienen te worden. Een dergelijke benadering zou uiteindelijk kunnen bijdragen aan meer geïndividuali-

seerde behandelingsstrategieën voor baarmoederhalskanker patiënten (47).

Concluderend worden in dit proefschrift nieuwe inzichten in het moleculaire mechanisme van lymfkliermetastasering, respons op (chemo)radiotherapie en prognose van baarmoederhalskanker gepresenteerd. Identificatie van celbiologische markers die respons op therapie kunnen voorspellen dienen te resulteren in het vinden van nieuwe aangrijpingspunten voor therapie. Uiteindelijk zou dit kunnen leiden tot verbetering van de behandeling van baarmoederhalskanker met daardoor een hogere overlevingskans.

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Liefste Ruurd, inmiddels zijn we 7,5 jaar bij elkaar; wat mij betreft gaan we het ver*tien*dubbelen!

Groningen, november 2010

Curriculum Vitae

Curriculum Vitae

Maartje Noordhuis, geboren op 17 januari 1986 in Groningen, groeide op in Zuurdijk (Groningen), waar haar ouders een akkerbouw bedrijf hadden. Op het Hogeland College in Warffum haalde zij in 2004 cum laude haar vwo diploma. Hierna begon zij met de studie geneeskunde aan de Rijksuniversiteit Groningen. Tijdens het 3e studiejaar startte zij met een onderzoeksproject bij de afdeling Gynaecologische Oncologie van het Universitair Medisch Centrum Groningen. In juli 2007 werd de Bachelor behaald, waarna zij de wetenschappelijke stage gedaan heeft bij deze afdeling. Aansluitend is zij in september 2008 gestart met promotieonderzoek in het kader van een MD/PhD-traject van de Junior Scientific Masterclass. Maartje heeft het eerste jaar van haar coschappen (junior coschappen) gelopen in het Martini Ziekenhuis te Groningen. Na haar promotie op 17 januari 2011 zal zij gedurende acht maanden gaan werken aan een onderzoeksproject in het laboratorium van prof. D. Sidransky, Johns Hopkins University School of Medicine, Baltimore, Maryland, U.S.A. Daarna zal zij de laatste 1,5 jaar van haar coschappen afronden.