

# **Biomarkers in chemoradiotherapy of cervical cancer:**

## **Focus on EGFR and DCE-MR imaging**

by

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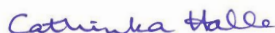
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## Abbreviations

aCGH	- array comparative genomic hybridization
AE	- adverse effects
AIF	- arterial input function
ATP	- adenosine triphosphate
BAC	- bacterial artificial chromosome
CIN	- cervical intraepithelial lesion
CT	- computed tomography
DCE-MRI	- dynamic contrast enhanced - magnetic resonance imaging
DNA	- deoxyribonucleic acid
DNA-PK	- DNA-dependent protein kinase
DSS	- disease specific survival
Gd-DTPA	- gadopentetate dimeglumine
ECD	- extracellular domain
EES	- extravascular-extracellular space
eGOn	- explore gene ontology
FDG	- 2' fluoro-2' deoxyglucose
FDR	- false discovery rate
FIGO	- International Federation of Gynecology and Obstetrics
<sup>18</sup> F-MISO	- <sup>18</sup> F-fluoromisonidazole
GO	- gene ontology
Gy	- gray
HGNC	- hugo gene nomenclature committee
HPV	- human papilloma virus
HR	- high risk
ICD	- intracellular domain
IHC	- immunohistochemistry
IMRT	- intensity-modulated radiation therapy
mRNA	- messenger ribonucleic acid
NHEJ	- non-homologous end joining
OXPHOS	- oxidative phosphorylation
PET	- positron emission tomography
PFS	- progression free survival

PLA	- proximity ligation assay
RECIST	- response evaluation criteria in solid tumors
RSI	- relative signal intensity
RT	- radiotherapy
RTK	- receptor tyrosine kinase
RT-PCR	- reverse transcription polymerase chain reaction
SAM-GS	- significance analysis of microarrays for gene sets
TCA	- tricarboxyl acid
TK	- tyrosine kinase
UPR	- unfolded protein response
5-FU	- 5-fluorouracile

## Gene identifications

<b>HGNC symbol</b>	<b>Gene name</b>
EGFR	- epidermal growth factor receptor
ERBB2	- v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
ERBB3	- v-erb-b2 erythroblastic leukemia viral oncogene homolog 3
ERBB4	- v-erb-a erythroblastic leukemia viral oncogene homolog 4
HIF1 $\alpha$	- hypoxia inducible factor 1, alpha subunit
MAX	- MYC associated factor X
MYC	- v-myc myelocytomatosis viral oncogene homolog
KIT	- v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
KRAS	- v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
PDGFR	- platelet-derived growth factor receptor
PI3K	- phosphoinositide-3-kinase
PTEN	- phosphatase and tensin homolog
RB1	- retinoblastoma protein
SRC	- v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)
STC2	- stanniocalcin 2
TP53	- tumor protein p53
VEGFR (KDR)	- kinase insert domain receptor (a type III receptor tyrosine kinase)
VHL	- von Hippel-Lindeau tumor-suppressor

## List of publications

- I. Halle C, Lando M, Sundfør K, Kristensen GB, Holm R, Lyng H. Phosphorylation of EGFR measured with in situ proximity ligation assay: Relationship to EGFR protein level and gene dosage in cervical cancer. *Radiother Oncol.* 2011, 101(1):152-7
- II. Halle C, Lando M, Svendsrud DH, Clancy T, Holden M, Sundfør K, Kristensen GB, Holm R, Lyng H. Membranous expression of ectodomain isoforms of the epidermal growth factor receptor predicts outcome after chemoradiotherapy of lymph node-negative cervical cancer. *Clinical Cancer Research*, 2011, 17(16):5501-12.
- III. Halle C, Andersen E, Lando M, Hasvold G, Holden M, Syljuåsen R, Sundfør K, Kristensen G, Holm R, Malinen E, Lyng H. Dynamic contrast enhanced - MR imaging portrays hypoxia induced gene expression in chemoradioresistant cervical cancer *Manuscript.*



## Aim of the thesis

There is a great need for biomarkers in the therapy of cervical cancer, in order to develop novel therapeutic strategies to improve the primary treatment and for treating recurrent disease. In addition to identifying potential biomarkers and their signaling pathways, it is important to develop applicable methods for use of the biomarkers in the clinic. Both of these aspects are studied in the current thesis.

EGFR has been suggested as a biomarker of aggressiveness in multiple cancer types; however, its potential in cervical cancer has not yet been established. Since therapeutics targeting EGFR has already been developed and approved for clinical use, it would clearly be beneficial to further explore the potential of EGFR as a biomarker in cervical cancer.

DCE-MRI has recently emerged as an exciting method to evaluate the biology and aggressiveness of a tumor, as an alternative or supplement to methods requiring tumor biopsies. Imaging is a well suited method for implementing the use of biomarkers in the clinic since it is already utilized in treatment strategies, and since it provides a non-invasive, repeatable method for assessment of tumor biology. However, the relation between various imaging parameters and the molecular properties of the tumor is currently not known. In this work, a prognostic DCE-MRI parameter was chosen to investigate to what extent it was correlated with a specific biological process and thereby could be used to depict biomarkers related to this phenotype.

The specific aims of this study were to:

1. Investigate to what extent EGFR may be used as a biomarker in cervical cancer by exploring the expression of the various protein isoforms together with the phosphorylation status in relation to cellular localization, gene dosage of EGFR, and treatment outcome of the patients.
2. Investigate whether DCE-MRI may be used to depict biomarkers or an aggressive phenotype by exploring the relationship between the prognostic DCE-MRI parameter  $A_{\text{Brix}}$  and gene expression of the tumor tissue.



# Introduction

## Cancer in general

It is believed that cancer arise from clonal evolution of one single cell (Nowell 1976), where genetic aberrations drive the gradual transformation of normal human cells into highly malignant derivatives in a stepwise manner (Hanahan and Weinberg 2011) (Fig. 1). The process has similarities with Darwinian evolution in that cells acquiring random aberrations and epigenetic changes that confer some kind of growth advantage, will obtain selective advantage over cells not having these changes. Such altered sub-clones of cells may thus be able to outgrow and further dominate their local tissue environment, eventually forming a cancerous tumor with invasive and/or metastatic properties.

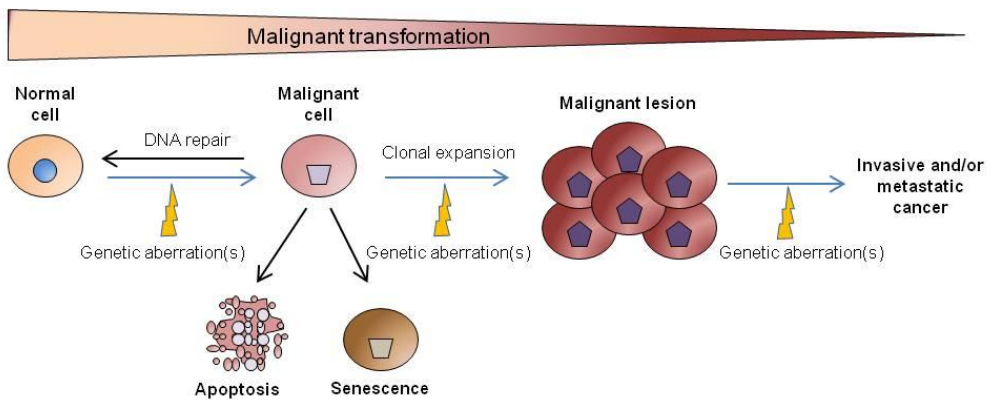


Figure 1: The stepwise process of carcinogenesis from a normal cell into a malignant tumor.

Most of the genetic aberrations are not hereditary, but arise as a result of damage to the DNA (Bertram 2000). This may occur as a consequence of endogenous stimuli such as free radicals generated during metabolism, or from extrinsic factors such as chemical carcinogens, virus infection, UV radiation, or ionizing radiation. Usually, the extremely efficient genome maintenance systems in the cells will detect and resolve defects in the DNA (Hanahan and Weinberg 2011), thereby counteracting the formation of genetic aberrations. Furthermore, cells in which the genomic defects cannot be repaired will normally undergo apoptosis or enter cellular senescence, thereby preventing the formation and/or growth of potential cancer cells

(Fig. 1). These mechanisms thus ensure a low rate of spontaneous mutations during each cell generation. Studies have shown that four to eight rate-limiting genetic aberrations may be required for the successive pathogenesis from a single cell into a malignant tumor, depending on cancer type (Renan 1993). A prevailing belief, although debated (Salk et al. 2010; Negrini et al. 2010), is thus that endogenous elevation of the mutation rates, also referred to as genomic instability, is a prerequisite for the acquisition of the multiple genetic aberrations which are needed during the process of carcinogenesis (Loeb 1991; Hartwell 1992).

### **The nature of genetic aberrations in cancer**

The genetic aberrations which provide the transforming cells with selective advantages include those that lead to activation of proto-oncogenes, or to inactivation of recessive tumor suppressor genes. The normal function of unaltered proto-oncogenes is to stimulate cell division and growth (Chial 2008). In contrast, the role of tumor suppressor genes is to restrain cell division and growth, and to help maintain genomic stability (Negrini et al. 2010). Thus, increased protein production from oncogenes or absence of tumor suppressor proteins caused by genetic aberrations will in general result in uncontrolled tissue proliferation and growth, and possibly to genomic instability.

The changes in the genome which affects these cancer genes may occur through multiple mechanisms (Lengauer et al. 1998). Minor changes in the DNA sequence may arise through substitutions of single nucleotides, so called point mutations (Fig. 2), or due to insertions or deletions of few nucleotides. This may affect the promoter region or the coding region of a certain gene, and further result in altered expression levels or change the function or stability of the gene. An example of a frequently mutated gene across several cancer types is the *KRAS* proto-oncogene, where point-mutations lead to constitutive activation of the protein encoded by the gene (Brink et al. 2003; Siegfried et al. 1997) (Fig. 2). Larger aberrations involve the gain or loss of whole chromosomes, which result in cells with an abnormal number of chromosomes, so called aneuploidy. This type of aberration is a feature of nearly all human cancers (Lengauer et al. 1998), and is often associated with gain of oncogenes or loss of tumor suppressor genes. This may be exemplified by the frequent loss of chromosome 10 and gain of chromosome 7 in glioblastomas, which contains among others the tumor suppressor gene *PTEN* and the oncogene *EGFR*, respectively (Wang et al. 1997; Romeike et al. 2001). A third type of aberration in

cancer is chromosomal translocations, where a segment from one chromosome is transferred to a new site on the same chromosome or to a non-homologous chromosome. This could lead to the recombination of coding regions from two different genes, which may result in expression of proteins with oncogenic properties. An example is the well known BCR-ABL fusion protein on the so called Philadelphia Chromosome, which results from a reciprocal translocation between chromosome 9 and 22 (Rowley 2001) (Fig. 2). The last common genetic aberration found in cancer is gene amplifications, where multiple copies of an “amplicon” of 0.5 – 10 megabases of DNA are seen, often containing a growth-promoting gene(s). Genomic regions containing various receptor tyrosine kinases (RTKs), respectively, are frequently found to be amplified in cancer, such as amplifications of the *epidermal growth factor (EGF)*- and *ERBB2-receptors* in breast and gastric tumors, among others (Gajria and Chandarlapaty 2011; Lorenzen and Lordick 2011; Lv et al. 2011) (Fig. 2).

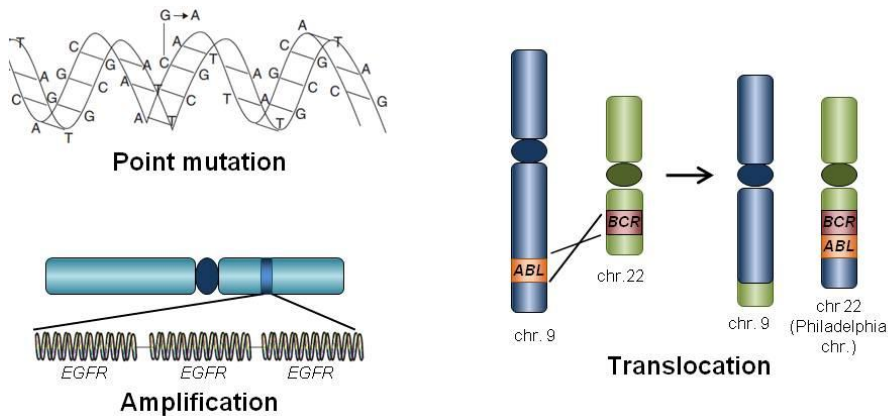


Figure 2: Some of the common genetic aberrations in cancer which may lead to oncogene activation.

It appears that the expression of many oncogenes and tumor suppressor genes are regulated not only through one, but through many of the above-mentioned mechanisms. One example is *EGFR*, which has been shown to harbor activating point mutations in some tumors, and/or be overexpressed due to either gain of the whole chromosome 7 or gain of an amplicon containing the *EGFR* gene in others (Lv et al. 2011). Furthermore, studies have indicated that *EGFR* may also be regulated through promoter methylation (Scartozzi et al. 2011), which is an epigenetic, non-mutational manner of regulating gene expression (Berdasco and Esteller 2010). Regardless

of the regulatory technique, the consequence seems to be constitutive activation of the receptor, with subsequent increase of tyrosine kinase (TK) signaling, enabling the cell to sustain proliferative signaling (Bubril and Yarden 2007). This variety of mechanisms for regulating one single gene illustrates the complexity of the genetic aberrations which is underlying malignant tumors. In addition to regulation on the genetic level, the expression and activity of a gene product may also be modulated on other levels, such as by microRNA binding to mRNA (Cui et al. 2006) or by post-transcriptional modification of proteins through phosphorylation or acetylation (Han and Martinage 1992), among various others.

### The functional capabilities of cancer (The Hallmarks)

It is believed that in order for a cell to fully transform into a cancerous cell, it needs to acquire a particular set of capabilities and traits as a result of the genetic aberrations, the so called “Hallmarks of Cancer”, first defined by Hanahan and Weinberg in 2000 (Hanahan and Weinberg 2000), with additional emerging hallmarks in the revised version (Hanahan and Weinberg 2011) (Fig. 3). Their theory is as follows; to be able to sustain increased proliferation and growth, cells must have the ability to evade the growth inhibitory signals which normally exist in the cellular surroundings, and to proliferate without any external growth stimulating signals. Further, to continue dividing even with mutations in crucial genes, they must be able to

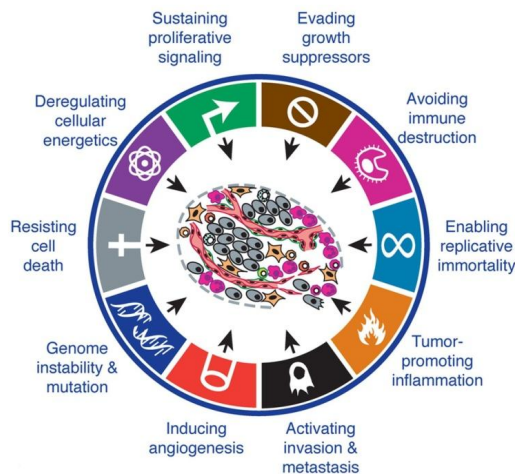


Figure 3: The Hallmarks of Cancer (Modified from Hanahan and Weinberg 2011)

resist cell death, which normally is induced in response to DNA damage. Additionally, the cells need to enable replicative immortality to divide eternally; normal cells can only divide a certain number of times because the chromosomal ends are shortened at each division. In order to avoid entering senescence after the limiting number of divisions, this process must be counteracted by the cells through one of various existing mechanisms. Moreover, for the cancerous cells to form a large tumor, an increased supply of oxygen and nutrients is needed. Thus, the cells need to induce the growth of new blood vessels into the tumor in order to secure this supply, a process called angiogenesis. Finally, the hallmark which has been described as the only one that really distinguishes malignant tumors from benign tumors (Lazebnik 2010), is the ability to invade surrounding tissue and metastasize to other tissues. Recently, two emerging hallmarks were added to the list of capabilities (Hanahan and Weinberg 2011), which are not yet generalized and fully validated. One is called “reprogramming of energy metabolism in the tumor”; in order to meet the energetic requirements from the increased growth and division of cells, the energy metabolism must be reprogrammed by limiting it largely to glycolysis. The second emerging hallmark is “evading immune destruction”; tumor cells need to avoid being detected by the immune system, and/or limit the degree of immunological killing. In 2011, Hanahan and Weinberg also described two emerging enabling *characteristics* for the hallmark capabilities. Firstly, bioactive molecules supplied to the tumor microenvironment by immune cells infiltrating the tumors may contribute to acquiring the other hallmarks, thus “tumor-promoting inflammation” has been added as an enabling characteristic. Additionally, it is known that many tumor cells are more genetically instable than normal cells, and “genome instability and mutation” was suggested as an enabling characteristic for the hallmarks of cancer, since this trait may be necessary to allow the evolving cell populations to reach the other biological capabilities.

## **Cervical cancer**

### **Epidemiology and aetiology**

Cervical cancer is the third most common cancer in women worldwide, developing annually in around 500 000 women and causing about 270 000 deaths (IARC, WHO 2010). Annually, there are almost 60000 incident cervical cancer cases and 30000 deaths in the whole of Europe. Owing to the organized cervical cytological screening, the incidence and mortality rates of cervical cancer have been greatly reduced in Western Europe (Arbyn et al. 2009; Laara et al. 1987; Sasieni et al. 1995; Peto et al. 2004; Lynge et al. 1989; Lazcano-Ponce et al. 2008). For women aged 35-64 in the UK, the risk of cervical cancer over the next five years was reduced by as much as 60-80% by participating in the UK cervical screening program, while the risk of advanced cervical cancer was reduced by about 90% (Sasieni et al. 2009). In developing countries, however, there is a lack of effective screening programs, and the recent improvements in the treatment of the disease are not available. Consequently, about 88% of the deaths from cervical cancer occur in these countries (IARC, WHO 2010).

There are numerous factors which have been shown to contribute significantly to the development of pre-invasive (cervical intraepithelial lesions (CIN)) and invasive cervical cancer. The major aetiological risk factor, which outweighs the others by far, is human papilloma virus (HPV) infection (Schiffman et al. 1993; Brisson et al. 1994). More than 99 % of all cervical cancers are linked to previous infection with the HPV (Walboomers et al. 1999). However, the probability of HPV persistence and progression to cervical neoplasia is increased by a number of host and environmental factors. The risk of developing CIN and invasive cancer of the cervix increases 5 to 10- fold by impairment of the immune system, whether this is due to immunosuppressive treatments (Birkeland et al. 1995) or human immunodeficiency (HIV) infection (Franceschi et al. 1998). In addition, the relative risk of developing cervical cancer is increased by certain sexually transmitted infections (Smith et al. 2002), long-term use of oral contraceptives (Moreno et al. 2002), high parity (Munoz et al. 2002), and tobacco smoking (Wyatt et al. 2001). Thus, the highest incidences of cervical cancer are found in populations where screening rates are still low, in combination with a high background prevalence of HPV infection and who have quite tolerant attitudes towards sexual behavior.



## **HPV and development of cervical cancer**

The HPV virus is transmitted by sexual contact, and HPV infection occurs in up to 75% of sexually active women at some time point (Syrjanen et al. 1990; Koutsky 1997). Even though HPV infections are common, most women never experience any symptoms, and the infection is normally cleared through an effective immune response within 6-12 months (zur Hausen 2002). Nonetheless, a small subset of the infections will progress to carcinoma *in situ* and finally to invasive cervical cancer. Over 100 different HPV types are currently known, and approximately 40 of these infect the squamous epithelium of the genital tract (de Villiers et al. 2004). The genital HPVs are divided into “low-risk” or “high-risk” (HR) types, based on their association with benign lesions or with pre-cancerous or cancerous lesions of the cervix, respectively. HPV 16 and 18, which belong to the HR group, are considered the most dangerous types, as they are found in about 70% of all cervical cancers. HPV-18 is mainly a risk factor for the development of adenocarcinomas (Bulk et al. 2006), and has been associated with poorly differentiated carcinomas with increased occurrence of lymph node involvement. HPV-16 on the other hand, is associated with both squamous cell carcinomas and adenocarcinomas.

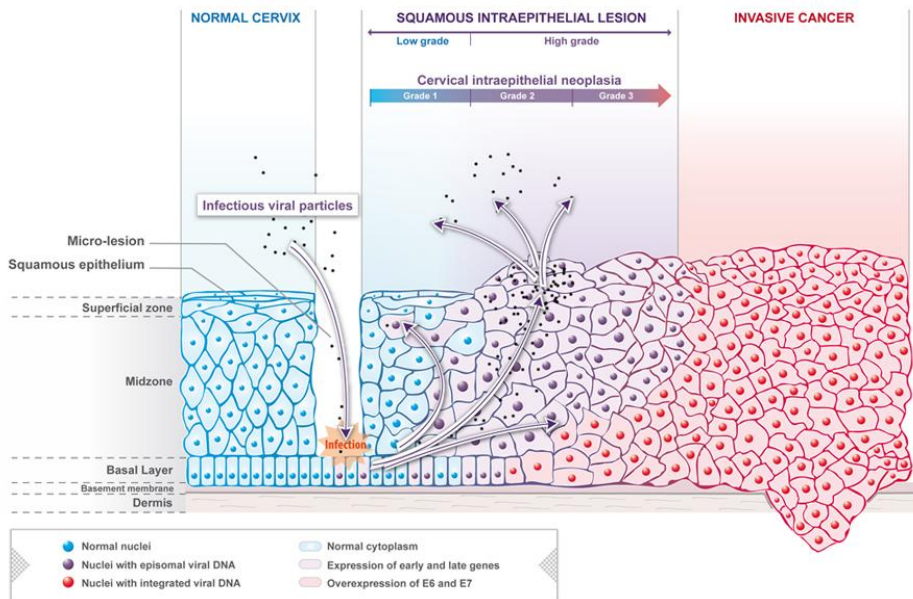


Figure 4: The sequential steps of HPV disease progression. Normal uninfected cervical epithelium is shown to the left, while the gradually infected epithelium is shown to the right, with the corresponding discrete cervical lesions marked on top. Infection of HPV occurs in the basal cells of the cervical epithelium, when the HPV genome is established in the nucleus as low-copy episomes, and early viral genes are expressed. After several years, the combined effect of the E6 and E7 oncoproteins and of the viral integration leads to carcinoma *in situ* and eventually to invasive carcinoma. (Courtesy of Gencitel 2011)

HPV infection occurs in the cells in the basal layer of the squamous epithelium, which are the only proliferating cells in normal epithelia (Doorbar 2006) (Fig. 4). Normally, these keratinocytes exit the cell cycle as they start to migrate up the epithelial layers and begin to differentiate. In the HPV-infected cells, however, the migrating cells remain active in the cell cycle as they reach the suprabasal layer, and terminal differentiation does not occur (Sherman et al. 1997). The viral genome is established as a stable episome in the infected cells at 50-100 copies per cell, which replicates together with the cellular DNA (Doorbar 2006; Stubenrauch and Laimins 1999). The viral proteins E1 and E2 play several roles in the early infection, such as initiating replication of the viral genome, and repressing early gene expression (Wilson et al. 2002; Steger et al. 1996; Thierry et al. 1987). E6 and E7 are the viral oncoproteins, responsible

for the increased proliferation of the basal epithelial cells (Massimi and Banks 1997; Scheffner et al. 1993). E7 binds retinoblastoma protein (RB1) and targets it for degradation, ultimately leading to transcription of genes associated with entry into S-phase. The primary role for the HR E6 protein is to target the tumor suppressor protein TP53 for degradation, in order to prevent growth arrest or apoptosis in response to E7-mediated cell cycle entry in the upper epithelial layers. In cells with persistent infection, the abrogation of several cell cycle checkpoints in response to E6 and E7 may also lead to accumulation of mutations and chromosomal instability, causing progression of cancer (Moody and Laimins 2010). In addition to giving the cells a proliferative advantage and contributing to genomic instability, the E6 protein activates telomerase (Howie et al. 2009), which is a critical step in immortalization and thus transformation of the cell, as previously mentioned.

While most of the HPV genomes persist in an episomal state in precancerous lesions, they are found to be integrated into the host genome in many high grade lesions (Moody and Laimins 2010). Integration usually disrupts the expression of E2, and since this protein may repress E6 and E7 genes in lesions with episomes, integration leads to deregulation of E6 and E7 and consequently to increased proliferation. Cells expressing E6 and E7 mRNA from integrated copies of the genome are consequently shown to provide the cells with a selective growth advantage as compared with cells containing episomes only of the HPV genome (Jeon et al. 1995; Jeon and Lambert 1995).

### **Histological subtypes**

There are two main types of cervical carcinoma, which are classified based on the cancerous cells' origin. Squamous cell carcinoma develops in the squamous cells that cover the surface of the ectocervix and transformation zone, and accounts for around 80% of invasive cervical cancer (Eifel et al. 1995; Smith et al. 2000). The squamous neoplasms are categorized as large cell keratinizing, large cell nonkeratinizing, or small cell carcinoma (Eifel et al. 2005). Adenocarcinoma is the other common subtype, which develops in the gland cells within the endocervix, and accounts for about 20 % of the cervical carcinomas (Waggoner 2003). However, the incidence of adenocarcinomas is rising in relation to that of squamous carcinoma in more developed countries (Waggoner 2003; Smith et al. 2000). Some cervical cancers may have features of both cell types, and are called adenosquamous carcinomas, which accounts for

fewer than 5% of the adenocarcinomas. A majority of studies have shown that the prognosis of adenocarcinomas is less favorable than that of squamous carcinomas, with a difference of 10-20% in 5-year overall survival (Davy et al. 2003; Eifel et al. 1995; Irie et al. 2000; Chen et al. 1999; Hopkins and Morley 1991; Nakanishi et al. 2000).

### **Disease dissemination**

The primary routes of spread for cervical carcinomas are by direct local extension into surrounding tissue or through the lymphatics to the pelvic and para-aortic lymph nodes (Gallup 2008). The direct extension usually involves the parametrium, which is the connective tissue between the layers of the broad ligament, eventually affecting the cardinal ligament. Further, it may spread to involve various parts of the vagina, and in more advanced cases, the tumor may spread posteriorly to involve the rectum or uterosacral ligaments or may invade the bladder (Camisão et al. 2007).

The lymphatic drainage of the cervix occurs through three main pathways; namely the lateral route along the external iliac vessels, the hypogastric route along the internal iliac vessels, and the presacral route along the uterosacral ligament (Park et al. 1994) (Fig. 5). All of these routes lead to the common iliac nodes, from where the tumor may spread to the paraaortic nodes. Normally, the lymphatic spread occurs through an orderly pattern where the paracervical and parametrial lymph nodes are the first to be affected, followed by the obturator, and the external and internal iliac nodes. These lymph nodes are thus called the primary nodal group. Further, the secondary nodal group is involved, which consists of the sacral, common iliac, inguinal, and paraaortic nodes. Detection of pathological lymph nodes may be performed through a sentinel lymph node procedure (Rasty et al. 2009). They may also, as in our study, be detected by magnetic resonance imaging (MRI) or computed tomography (CT) (Chung et al. 2010), where a lymph node is classified as pathologic whenever the short axis is equal to or exceeded 10mm at the time of diagnosis, according to the response evaluation criteria in solid tumors (RECIST) version 1.1 (van Persijn van Meerten EL et al. 2010).

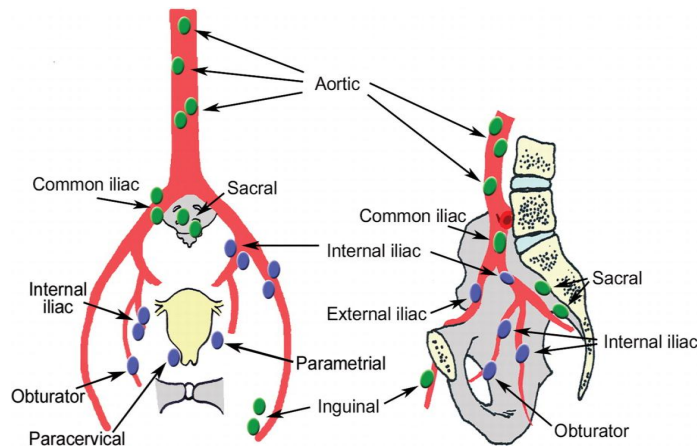


Figure 5 - Distribution of lymph node metastasis in patients with cervical cancer, with the most primarily affected lymph nodes in blue, and the secondary affected in green. (From Jeong et al. 2003)

Distant metastases from cervical tumors are primarily due to recurrent disease, and most commonly involve the liver, lung, bone, and/or extrapelvic nodes (Fagundes et al. 1992; Fulcher et al. 1999). It has been shown that the incidence of distant metastases is correlated with increasing stage, and that endometrial extension of the tumor and pelvic tumor control are other factors that can indicate distant dissemination.

## Staging

Staging of invasive carcinoma is determined clinically at the time of primary diagnosis (Waggoner 2003). In contrast to the TNM system that is used for staging of many other cancer types, the FIGO (Federation of International Gynecologists and Obstetricians) staging system does not include the presence of lymph node metastases, but is based mainly on the size of the malignant tumor in the cervix or its extension into the pelvis. According to FIGO, cervical cancer is staged into 4 different stages, with 10 substages, from IA to IVB (Pecorelli 2009). (Fig. 6) For stage I, the cancerous cells are strictly confined to the cervix. When the carcinoma has started to spread beyond the uterus, but not to the pelvic wall or lower part of the vagina, it is classified as stage II. Further, stage III describes a malignant tumor that extends to the lower part of the vagina or has spread to the muscles and ligaments that line the pelvic wall. In stage IV, the carcinoma is advanced, and has extended beyond the true pelvis or has involved the mucosa of the rectum or bladder.

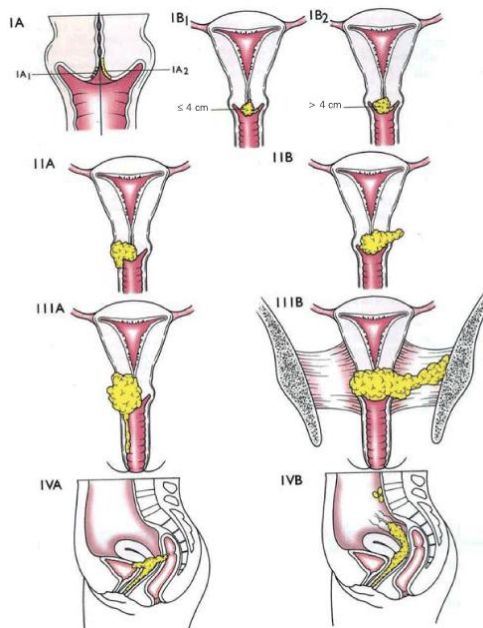


Figure 6: The FIGO stages of cervical cancer. (From Camisão et al. 2007)

### Current therapy and prognosis of cervical cancer

While the 5-year survival rate in Norway of cervical cancer in general is 73%, there is a great difference between the various stages, from 89% for stage I to only 18% for stage IV (Cancer Registry of Norway 2008) (Fig. 7). Early stages of cervical cancer are normally treated either surgically, including radical hysterectomy or pelvic lymph node dissection, or by a combination of chemotherapy and radiation (Rasty et al. 2009). For the small stage IA cervical cancers, surgery is the common treatment. Patients with locally advanced cervical cancers (stage IB2 - IVA) and patients with extra-cervical disease such as lymph node metastases, are primarily treated with concurrent chemoradiation comprising external beam irradiation and brachytherapy in combination with platinum-based chemotherapy (Movva et al. 2009; Rasty et al. 2009; Klopp and Eifel 2011). If lymph nodes exceeding 10mm are found in the pelvic wall, an additional boost of radiation is given to these areas (Kristensen, G.B. 2011). In case of metastases to the common iliac or para-aortic nodes (Figure 5), extended-field radiation to cover these nodes is given. Patients with stage IVB are not given the standard treatment, but

are treated individually, usually with 5FU followed by surgery or conventional radiotherapy as for the other stages.

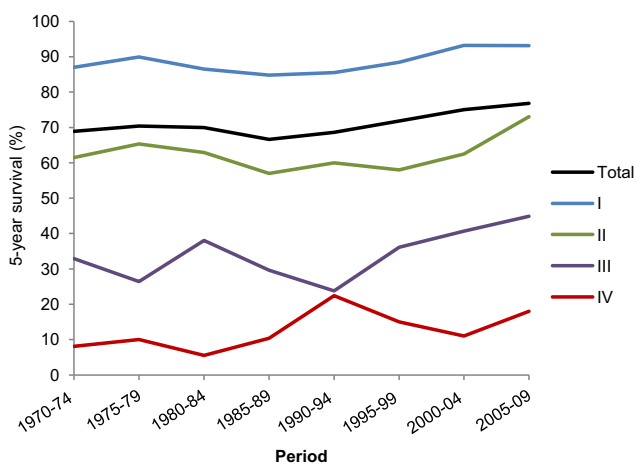


Figure 7: The 5-year survival for cervical cancer patients, with regard to time of diagnosis and stage (Adapted from Kristensen, G.B. 2011).

The rationale for using brachytherapy as part of the standard treatment, is that a high dose of radiation may be delivered to the tumor with a relatively low dose to the adjacent bladder and rectum, due to the steep dose gradient achieved with brachytherapy (Klopp and Eifel 2011). Additionally, due to movement of the brachytherapy source with the target, it has the capacity to overcome the limitations of external beam radiation caused by internal organ motion. The reason why concurrent platinum-based chemotherapy is added to the therapy regime, is that it has been shown to extend overall survival by an absolute 5-20 % as compared to radiation therapy alone, while the risk of pelvic recurrence is reduced by approximately 50% (Eifel et al. 2004).

As demonstrated in figure 7, the rates of survival in cervical cancer patients are negatively correlated with FIGO stage. However, a number of other tumor characteristics that are not included in the staging system also have influence on the prognosis. The volume of the tumor, as determined with high accuracy using imaging techniques (in particular MRI) (Oellinger et al.

2000; Chiang and Quek 2003), is strongly correlated with prognosis for patients treated with radiation or surgery (Perez et al. 1992; Kristensen et al. 1999). Another factor that influences the survival of cervical cancer patients is the presence of lymph node metastases. The incidence of metastases to the lymph nodes correlates with other parameters of poor prognosis such as increasing stage, tumor diameter, lymphovascular space involvement, and parametrial involvement (Kamura et al. 1999; Michel et al. 1998; Berman et al. 1984), and the presence of positive lymph nodes is one of the most important independent prognostic factors for cervical cancer (Creasman and Kohler 2004). The number of positive lymph nodes, and the site and number of nodal sites involved are also of prognostic significance (Ishikawa et al. 1999; Shigematsu et al. 1997).

In addition to the response rates, the incidence of serious side effects after therapy is an important aspect of the treatment regimes. Due to the anatomical location of the cervix in the pelvis, the lower ureteres, bladder and posterior urethra are exposed to radiation during treatment for cervical cancer. This may give rise to several urinary adverse effects (AEs) (Elliott and Malaeb 2011) and the probability of developing grade 1 and 2 AEs following RT for cervical cancer has been reported to be 28%, increasing by an additional 17.4% at 5 years. While the acute toxic effects of treatment generally are of short duration and may be resolved with medical management, the long term toxic effects may permanently impair the quality of life of the survivors. Thus, since the rate of recurrence is relatively high and the incidence of side effects is quite frequent, there is still a great need for improved treatment strategies. Several studies have tested the combination of additional chemotherapeutics with the current chemoradiation treatment. They have shown some promising effects, however, the overlapping toxicity between cisplatin and these therapeutic drugs approaches the limits of haematologic tolerance (Klopp and Eifel 2011). The identification of biomarkers or molecular targets for therapy in cervical tumors that may be used in personalized treatment strategies could potentially help improve the current therapy of cervical cancer and thereby contribute to better response rates, as well as to avoid additional side effects for the patients.



## **Biomarkers and molecular targets in cervical cancer**

In the field of cancer, a biomarker may be defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Biomarkers Definitions Working Group 2001), or more simplified, as anything that can be used to indicate a biological state such as a disease state. While diagnostic biomarkers indicate if a disease is already present in patients, predictive biomarkers give an indication of the probable effect of a given treatment on a patient, and prognostic biomarkers show the likely course of a disease regardless of treatment. Importantly, overlaps exist between these categories, in that most predictive factors have prognostic value (Rodriguez-Enriquez et al. 2011). Biomarkers may also be classified on characteristics, such as imaging biomarkers, or molecular biomarkers. Molecular biomarkers could be tumor associated proteins, mRNA or DNA fragments, which are used either individually or in signatures of multiple molecules. Imaging biomarkers are anatomical, physiological, biochemical, or molecular characteristics which are detectable by certain features or parameters from imaging modalities such as MRI, Positron Emission Tomography (PET) or CT (Smith et al. 2003).

The advantages of finding new biomarkers which may be used in the course of cancer treatment are many. As mentioned above, they may be used to improve diagnosis, and to predict response both to current treatment regimes and to novel molecularly targeted agents. Importantly, the use of biomarkers allows for more personalized treatment, since the biomarkers may be differentially present between patients (Eifel 2006). Biomarkers may also be used to identify appropriate patient groups for clinical trials, increasing the probability of new and efficient drugs proceeding to the clinic. An example of a well studied prognostic and predictive biomarker is the *ERBB2 (HER2/neu)* DNA amplification, which is accepted in the clinical practice to predict response to treatment with trastuzumab in breast cancer (Buyse et al. 2010). The glycoprotein CA-125 is another example of a protein which has been approved by the Food and Drug Administration (FDA) as a molecular biomarker for use in the clinic to pre-visit prognosis after treatment and for detecting disease recurrence of ovarian cancer (Rhea and Molinaro 2011). However, compared with the more than thousands of candidate biomarkers for various cancer types that are suggested in the literature, very few cancer biomarkers are

currently approved and implemented in the clinic (Rhea and Molinaro 2011; Polanski and Anderson 2007; Rodriguez-Enriquez et al. 2011). A major reason for this so called “pipeline problem”, is a lack of money, i.e. will of investment, as well as a lack of samples for testing and validating the biomarkers (Phillips et al. 2006), since an abundance of samples is critical to determine both validity and utility of novel biomarkers. Moreover, for the various targeted molecular drugs that have been developed against certain biomarkers, the rate of therapeutic success has been disappointingly poor (Faratian et al. 2009; Polanski and Anderson 2007). This observation may indicate that the probability of curing a certain cancer type through targeting of only one master biomarker, or similarly to predict response based on the expression level of one single protein, may be quite small. Additionally, it may suggest that we still need an improved understanding of the biology of these biomarkers in relation to the aggressiveness in the tumors, to be able to exploit the potential of the biomarkers. However, signatures based on the expression of multiple genes might have more potential, as they may provide more sensitivity in reflecting the aggressive properties of the tumor and consequently resistance to treatment.

### **Receptor tyrosine kinases as biomarkers in cervical cancer**

Receptor tyrosine kinases (RTKs) constitute a subclass of cell surface growth factor receptors which have intrinsic tyrosine kinase (TK) activity that is mainly controlled by various ligands (Gschwind et al. 2004). RTKs regulate a variety of functions in normal cells, and thus have important physiological functions. Deregulation of these proteins is a common event in cancer, and they have consequently been the subject of intense investigation with the prospective of developing targeted therapeutics directed against them. The mechanism of action of these proteins may be simplified as follows (Hubbard and Miller 2007); A relevant ligand binds to its receptor TK, inducing receptor dimerization followed by cross phosphorylation and activation of the receptors. A variety of cytoplasmic proteins is further phosphorylated by the activated receptors, leading to a cascade of events which eventually triggers the activation of transcription factors in the nucleus. The synthesis of mRNA and thus proteins is consequently increased, which ultimately leads to either growth or differentiation.

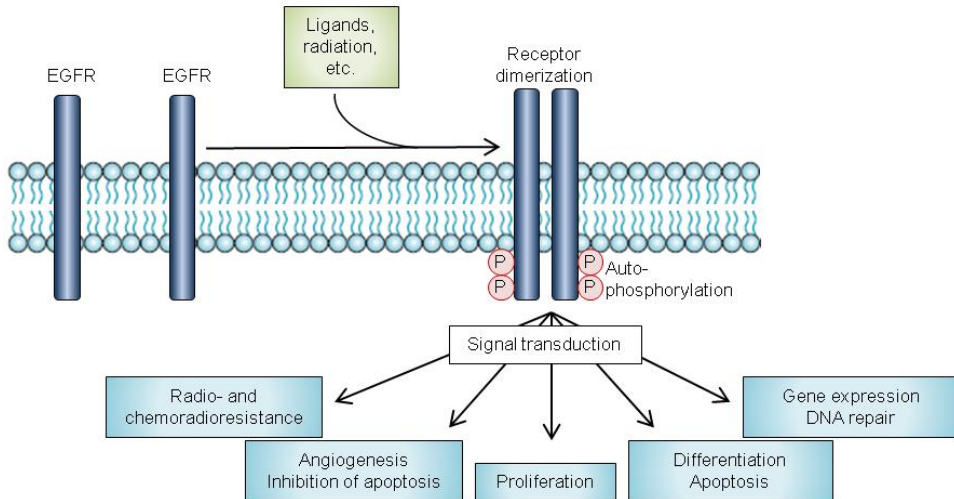
Several of the RTKs are attractive targets for directed therapies, such as VEGFR (KDR), PDGFR, and EGFR (Gschwind et al. 2004). The main role of VEGFR in tumors is to support

growth by facilitating the formation of new blood vessels, and a wide range of strategies for targeting VEGFR-mediated angiogenesis has been developed (Saharinen et al. 2011). Pre-clinical results have shown strong effects on reducing tumor size, blood vessel density, and tumor metastasis, and several inhibitors of VEGFR are currently approved for clinical use. With regard to cervical cancer, the VEGFR inhibitor pazopanib (GW786034; GlaxoSmithKline, London, UK) was recently evaluated in a phase II clinical trial, showing promising results (Monk and Pandite 2011). Pazopanib also inhibits PDGFR, a TK receptor which mainly activates proliferation and migration of cells. Only a few studies have investigated the role of PDGFR in cervical carcinogenesis, however, *in vitro* and pre-clinical studies have shown significant therapeutic effects of the PDGFR inhibitor Imatinib (Gleevec, Glivec, STI751) (Taja-Chayeb et al. 2006; Kummel et al. 2008). Finally, EGFR is one of the most extensively studied TK receptors, and its potential as a candidate for targeted therapy of cervical cancer is discussed in more detail below.

### ***The biology of EGFR and its current status as a biomarker in cervical cancer***

EGFR is a member of the oncogenic ERBB-family of RTKs, together with ERBB2, ERBB3, and ERBB4 (Wells 1999). Binding of one of the many EGFR ligands to the extracellular domain (ECD) of the receptor leads to the formation of a receptor dimer consisting of either two EGFR proteins (homodimerization), or of EGFR and another member of the ERBB-family (heterodimerization) (Fig. 8). This stimulates the intrinsic TK activity of the receptor, leading to autophosphorylation at multiple residues of the intracellular domain (ICD), and further activation of multiple signaling pathways such as the Ras-MAPK signaling cascade or the PI3K pathway. This in turn influences multiple biological processes such as cell cycle, proliferation, differentiation, motility, and cell death or survival. Overexpression and/or increased EGFR activity has been linked with resistance to both chemotherapy and radiation in tumors, and thereby to poor prognosis of the patients (Rodemann et al. 2007). EGFR and its downstream signaling networks are important parts of the cellular response to radiation exposure, in that EGFR may be activated by ionizing radiation even in the absence of a ligand (Dent et al. 2003; Yacoub et al. 2006; Schmidt-Ullrich et al. 1997). There seems to be mainly two means by which EGFR contributes to chemoradioresistance, namely through inhibition of apoptosis through the PI3K-Akt pathway and by promotion of cell proliferation through Ras-MAPK (Schmidt-Ullrich et al. 1997). Additionally, upon DNA damage following radiation and heat

induced stress, EGFR translocates to the nucleus where it associates with DNA-PK, leading to DNA repair via non homologous end joining (NHEJ) and consequently to radioresistance (Lo and Hung 2006). EGFR may also contribute to DNA repair through other mechanisms such as by influencing the expression of genes involved in base excision repair (BER) (Yacoub et al. 2003).



**Figure 8:** Simplified overview of EGFR activation, dimerization and signal transduction, as well as some of the affected biological processes.

EGFR is shown to be frequently overexpressed in several tumor types, including cervical carcinomas (Ngan et al. 2001; Yamashita et al. 2009). However, the relationship between EGFR overexpression and survival of cervical cancer patients is not clear, as studies have shown inconsistent results (Soonthornthum et al. 2011). In addition, the biology of EGFR is complex and not yet completely understood. EGFR is well known for its activation of various signaling pathways starting with the phosphorylation of its TK domain. However, it was recently shown that a TK independent role of EGFR may be important for the survival of cancer cells (Weihua et al. 2008), highlighting the intricacy of understanding the mechanisms of action of certain oncogenes. In the clinic, EGFR may either be targeted with anti-EGFR monoclonal antibodies directed towards the ECD of the receptor, or with TK inhibitors, which specifically inhibits its TK domain. Various agents targeting EGFR have shown to be efficient

in clinical trials of lung, colon, pancreas, and head and neck cancers (Sobrero et al. 2008; Rivera et al. 2009; Senderowicz et al. 2007; Johnson et al. 2005). However, not all patients benefit from this targeting, and attempts to define a common predictor of response between the different tumor types for these agents have not been successful. The wild type status of *KRAS* is predictive of response to the monoclonal antibodies cetuximab and panitumumab in colon cancer (Karapetis et al. 2008; Amado et al. 2008), and both *KRAS* mutations and specific mutations in the TK domain of EGFR are associated with response to erlotinib in lung cancer (Eberhard et al. 2005). In cervical cancer, however, very few cases of mutations in *KRAS* have been found, and mutations in the TK domain of EGFR are also rare (Arias-Pulido et al. 2008; Iida et al. 2011; Pochylski and Kwasniewska 2003; Stenzel et al. 2001). Nonetheless, both cetuximab and erlotinib are currently being investigated in combination with the standard chemoradiotherapy in clinical trials of cervical cancer patients. Recently, two phase II studies investigating the effect of cetuximab was performed in recurrent or persistent carcinoma of the cervix, but limited activity of the monoclonal antibody was found (Santin et al. 2011; Farley et al. 2011). The same lack of effect was demonstrated for the TK inhibitor erlotinib in a phase II trial of recurrent squamous cell carcinoma of the cervix (Schilder et al. 2009). It is thus evident that further knowledge about the mechanisms of action of EGFR in cervical tumors is needed in order to select cervical cancer patients for EGFR targeted therapy and to elucidate its potential as a target in cervical tumors. A deeper understanding of the biology of EGFR is also necessary to potentially allow the use of EGFR as a marker of aggressiveness, i.e. as a prognostic marker, in cervical cancer.

### **The use of functional and molecular imaging in the field of biomarkers**

The recent advances in “functional and molecular imaging” technologies (see panel 1), such as MRI and PET, might significantly impact the field of molecular biomarkers and personalized therapy. While such imaging modalities were previously focused on depicting the anatomy of tumors, their potential to measure tissue function as well as expression of specific phenotypes or even molecules is becoming increasingly recognized (Stephen and Gillies 2007).

There are many advantages in using functional and molecular imaging to complement or replace traditional tissue sampling. While the need of tumor biopsies necessitates an invasive procedure, which may cause discomfort for the patient and as well as being time-consuming for the medical practitioners, imaging provides non-invasive assessment of various parameters such as angiogenesis and metabolic features. Furthermore, while traditional methods for assessing biomarkers often relies on semi-quantitative estimations which are influenced by the evaluation and choice of a cut-off level by a pathologist (Rodriguez-Enriquez et al. 2011), functional and

**Molecular imaging:** Imaging of tracers or contrast agents which interact with tissue in a molecularly specific fashion.

**Functional imaging:** Endogenous or exogenous contrast is depicted to provide information on tissue behavior or phenotype.

**Functional and molecular imaging:** Comprises both of the above-mentioned, as their demarcation is ill-defined (Stephen and Gillies 2007)

Panel 1: Clarification of the term “functional and molecular imaging“

molecular imaging may contribute with qualitative and quantitative objective data regarding parameters of interest. Since imaging gives a picture of the whole tumor, the imaging modalities may also better represent the heterogeneity of the tumor compared with the limited number of biopsies which are normally analyzed from each tumor. In addition, functional and molecular imaging may be used to get longitudinal information about the metabolism and pathophysiology of individual tumors during the course of treatment, as they allow for repeated non-destructive measurements of tumors. Finally, it also offers the potential application to guide intensity modulated radiation therapy (IMRT) (Geets et al. 2007), to spare adjacent normal tissue for high doses of radiotherapy, or to provide higher doses to specific areas of the tumors identified by the imaging techniques. To be able to fully exploit the functional and molecular imaging techniques for these purposes, it is necessary to explore the functional and molecular background of the various imaging parameters. Only a small number of studies have tried to correlate biomarker imaging with expression or activity of specific genes or pathways that are targeted with particular drugs (Serganova et al. 2008). However, it is becoming

increasingly recognized that several molecular and functional imaging parameters may reflect underlying gene expression patterns in various cancer types, a phenomenon sometimes termed “radiogenomics” (Rutman and Kuo 2009; Padhani and Miles 2010). This is an area of great interest since it would be highly advantageous to use non-invasive imaging techniques when developing and assessing new drugs against genes with aberrations such as *EGFR*, *TP53* and *HIF1A*, or against aggressive phenotypes like increased proliferation and hypoxia in tumors. Many of the parameters from the various imaging technologies lack consistent biological or molecular correlates, thus a large amount of information encoded in imaging studies is currently uncharacterized and consequently unexploited. It has been hypothesized that the cost of clinical trials could be greatly reduced if functional and molecular imaging was employed to increase the efficiency of the trial process (Stephen and Gillies 2007). Since the current cost of research and development per new approved drug is estimated to be approximately \$1.6 billion, this is clearly an area worth improving.

### ***DCE-MRI and its current use in cancer therapy***

In the treatment of cervical cancer patients, MRI is utilized to obtain information about the anatomy of the tumor, and has been proven useful in determining the size of the cervical neoplasm, as well as in detecting parametrial invasion, and bladder or rectal invasion (Follen et al. 2003). The presence and consistency of enlarged lymph nodes, obstruction of the ureter, and lung or liver metastases may also be detected using MRI. Since MRI is already used in the management of cervical cancer, Dynamic contrast enhanced (DCE)- MRI may easily be implemented as part of the treatment regime. DCE-MRI provides insight into biological properties of the tumor such as tumor perfusion, vessel permeability and the volume of the extravascular-extracellular space (EES) (Li et al. 2011). It is therefore one of the imaging modalities with a great potential to measure tumor response to cancer therapy, in particular to drugs that targets biological processes associated with angiogenesis or perfusion. Information about these parameters is achieved by acquiring serial MR images before, during and after administration of a tracer, which is often based on gadolinium, such as the Gd-DTPA (gadopentetate dimeglumine) (Knopp et al. 2001). The tracer is administered intravenously and will travel through the vascular system and immediately leak from the tumor vasculature and accumulate in the tumor. It will further re-diffuse back into the vascular system and eventually be eliminated via the urinary system. The kinetics of the wash-in and wash-out of the contrast

agent may be analyzed pixel-by-pixel from the MR time series images. The behavior of the tracer may be described with descriptive tools such as relative signal intensity (RSI), and slope and rate of washout (Evelhoch 1999). However, to understand the underlying physiology of these descriptive parameters, pharmacokinetic (PK) modeling is necessary. The parameters provided by the PK models describe the association between the contrast enhancement data and the vascular anatomy and physiology of the tumor (Choyke et al. 2003). The Brix model is a commonly used “two compartment” PK model. Instead of assessing the arterial concentration which is necessary for other models, this model treats the vascular space as a reservoir with uniform concentration and a constant clearance rate (Brix et al. 1991). It is assumed that the vascular concentration curve results from a constant infusion of known duration of tracer into the vascular space. Since tumors are assumed to have a negligible vascular component, the concentration in the tumor may be described by the concentration in the EES. Given the assumptions made in the Brix model, a mathematical expression can be used to describe the EES concentration of contrast agent and hence tumor concentration in terms of three parameters:  $k_{el}$ ,  $k_{ep}$ , and  $A_{Brix}$ , as follows:

$$RSI(t) = A_{Brix} \frac{k_{ep}}{k_{el} - k_{ep}} (e^{-k_{ep}t} - e^{-k_{el}t}) \quad (1)$$

$k_{el}$  describes the clearance rate of the contrast agent from the vascular compartment, while  $k_{ep}$  is the rate constant of the contrast from the EES to the vascular compartment.  $A_{Brix}$  describes the concentration of tracer in the reservoir, and is a function of tracer dose, perfusion, vascular volume, extracellular volume and permeability. By fitting the model to the time series data from the DCE-MRI images,  $A_{Brix}$ ,  $k_{el}$ , and  $k_{ep}$  can be estimated.

One of the current uses of DCE-MRI is in the management of breast cancer, where it is employed to detect recurrent disease and investigate multifocal tumors in high risk patients (Brix et al. 2010). It is also utilized to improve characterization of several cancer types, and to detect cancer in the case of multiple myelomas. However, the potential of DCE-MRI to assess tumor response to targeted treatment is not yet exploited in the clinic. There are currently some obstacles to the application of this technique in the clinic, in that standardization of scan protocols and analysis methods are lacking. However, DCE-MRI also has some advantages over CT and PET (O'Connor et al. 2007) in that it does not involve ionizing radiation, it presents with a better spatial resolution, and it may be performed on standard 1.5 Tesla MRI



scanners. Moreover, the ability of DCE-MRI to describe the vascular properties of a tumor makes it advantageous for evaluating the response of anti-angiogenic and vascular targeting agents. Accordingly, about 30 studies of such agents have been reported to date, including both phase I and phase II studies (Jackson et al. 2007).

## Summary of the publications

### Publication I:

#### **Phosphorylation of EGFR measured with in situ proximity ligation assay: Relationship to EGFR protein level and gene dosage in cervical cancer**

This study was performed to characterize the expression of Tyr1068 phosphorylated epidermal growth factor receptor (EGFR) in relation to the EGFR protein level and gene dosage in cervical cancer. Pretreatment tumor biopsies from 178 patients were included in the study. The protein level of EGFR was assessed in all patients by conventional immunohistochemistry, while the phosphorylation of EGFR on Tyr1068 was detected with the sensitive and specific *in situ* proximity ligation assay (PLA) in 97 of the EGFR positive tumors. EGFR gene dosage was derived from array comparative genomic hybridization of 86 cases. We demonstrated that EGFR was expressed in most tumors, and the expression of EGFR was correlated with phosphorylated EGFR for both membrane and cytoplasm. However, the percentage of EGFR positive tumors was higher than the phosphorylated percentage, with only about half of the EGFR positive tumors displaying phosphorylated EGFR. Moreover, tumor regions with high levels of EGFR without phosphorylation were occasionally seen. While the protein level of EGFR was not correlated with gene dosage, an increase in the phosphorylation in both the membrane and the cytoplasm was seen in the 11 tumors with gain of EGFR. Thus, in contrast to gain of the EGFR chromosomal region, a high level of EGFR protein may not necessarily indicate Tyr1068 phosphorylation and thereby activation of the receptor in cervical cancer.

## **Publication II:**

### **Membranous Expression of Ectodomain Isoforms of the Epidermal Growth Factor Receptor Predicts Outcome after Chemoradiotherapy of Lymph Node Negative Cervical Cancer**

The aim of this study was to compare the prognostic significance of ectodomain isoforms of the epidermal growth factor receptor (EGFR), which lack the tyrosine kinase (TK) domain, with that of the full length receptor and its autophosphorylation status. 178 patients with squamous cell cervical carcinoma treated with conventional chemoradiotherapy were included in the study. Immunohistochemistry was applied to assess the expression of EGFR isoforms, and the detection of the various isoforms was confirmed with western blotting and RT-PCR. *In situ* proximity ligation assay was used to detect EGFR specific autophosphorylation. By the use of gene expression analysis with Illumina beadarrays, pathways associated with the expression of ectodomain isoforms were studied in 110 patients and validated in an independent cohort of 41 patients. Membranous expression of ectodomain isoforms alone, without the co-expression of the full length receptor, showed correlations to poor clinical outcome for all endpoints that were highly significant for lymph node negative patients and independent of clinical variables. The ectodomain EGFR isoforms appeared to be primarily 60kD products of alternative EGFR transcripts. The membranous expression of these isoforms alone was correlated with transcriptional regulation of oncogenic pathways, including activation of *MYC* and *MAX*, which was significantly associated with poor outcome. To confirm this aggressive phenotype of ectodomain EGFR expressing tumors, these results were confirmed in the independent cohort. Neither total nor full length EGFR protein level, nor autophosphorylation status showed prognostic significance. These findings indicate that membranous expression of ectodomain EGFR isoforms, and not TK activation, predicts poor outcome after chemoradiotherapy of patients with lymph node negative cervical cancer. It thus implies that targeted therapy aimed at inhibiting the TK activation of EGFR may not give the desired effect in cervical cancer patients. Further, it suggests that a deeper understanding of the biology and possible aggressive properties of the ectodomain isoforms is required to successfully target EGFR in cervical cancer.

### **Publication III:**

#### **Dynamic contrast enhanced- MR imaging depicts hypoxia induced gene expression in chemoradioresistant cervical cancer**

In this work, a previously developed percentile screening method was utilized to systematically evaluate the prognostic impact of the DCE-MRI parameter  $A_{\text{Brix}}$ . This parameter was further investigated in combination with gene expression profiling to explore the molecular phenotype underlying its aggressiveness. A total of 187 patients with adeno-, adenosquamous-, or squamous- carcinoma of the uterine cervix were included, all treated with curative chemoradiotherapy. DCE-MRI was performed for 78 of the patients. The Brix pharmacokinetic model was fitted to the temporal contrast enhancement pattern in each tumor voxel, and histogram analysis was performed to identify a prognostic  $A_{\text{Brix}}$  parameter. Tumors from 155 patients underwent gene expression profiling, including 46 of the DCE-MRI patients. The relation between  $A_{\text{Brix}}$  and the gene expression data for the 46 patients were investigated using both unsupervised gene ontology- and supervised gene set -analysis. For the gene set analysis, cervical cancer specific hypoxia gene sets were created from experiments where cervical cancer cell lines were kept for 24h in either normoxic or hypoxic (0.2% O<sub>2</sub>) conditions. Other phenotypes explored in the gene set analysis were proliferation, wound healing and radiation resistance, using previously published gene sets. Immunohistochemistry was performed to investigate expression of HIF1 $\alpha$  in the 32 remaining DCE-MRI patients. The gene ontology analysis revealed that the biological processes such as metabolism, DNA damage repair and cell cycle regulation were significantly associated with  $A_{\text{Brix}}$ , and the gene set analysis showed that hypoxia was the most significant phenotype associated with  $A_{\text{Brix}}$ . The relation between low  $A_{\text{Brix}}$  and hypoxia was confirmed in independent patients by the finding of a negative correlation between HIF1 $\alpha$  protein expression and  $A_{\text{Brix}}$ . Based on the hypoxia gene sets, a DCE-MRI hypoxia gene signature was created and demonstrated to have prognostic value in the DCE-MRI patients. The prognostic significance of the DCE-MRI hypoxia gene signature was further validated in an independent group of 109 patients, using clustering and a calculated hypoxia score based on the genes in the DCE-MRI hypoxia gene signature. This study thus demonstrated that the  $A_{\text{Brix}}$  parameter derived from pharmacokinetic analysis of DCE-MRI reflects an aggressive hypoxic phenotype of cervical tumors. This implies that DCE-MRI may be useful to non-invasively visualize tumor hypoxia and select patients with aggressive disease.

# Experimental considerations

## Patient material

The 236 patients included in this study (Table 1) were diagnosed with primary carcinoma of the uterine cervix and recruited to our chemoradiotherapy protocol at the Norwegian Radium Hospital from 1999 to 2009. Written-informed consent was acquired from all patients, and the study was approved by the regional committee of medical research ethics in southern Norway. Tumor stage (FIGO) was ranging from 1B2 through 4A.

Table 1: The number of patients included in the current study in relation to the methods they were assessed by is indicated, as well as the inclusion criteria for the various papers.

	Paper I	Paper II	Paper III	All papers
Inclusion criteria	Squamous histology + paraffin sections for IHC	Squamous histology	1. DCE-MRI and/or Illumina-data 2. paraffin sections	
Total no of patients	178	219	187	236
DCE-MRI	↓	↓	↓ 78	78
IHC	↓ 178	↓ 178	↙ 32	191
PLA	↘ 97	↘ 97	↓	97
Illumina	↓	(39 + 71) + 41	↓ 46 + 109	155
aCGH	↓ (14 + 72)			86

Note: The numbers of patients within a parenthesis were used in the same round of analysis. Abbreviations: DCE-MRI, dynamic contrast enhanced-magnetic resonance imaging; IHC, immunohistochemistry; PLA, proximity ligation assay; aCGH, array comparative genomic hybridization

MRI, or in a few cases CT, was used to detect pathological lymph nodes in the pelvis at the time of diagnosis. All patients were treated with external radiation of 50 grey (Gy) to tumor, parametria, and adjacent pelvic wall and 45 Gy to the rest of the pelvis in 25 fractions. Additionally, 21 Gy in five fractions were given by endocavitary brachytherapy to point A.

During the period of external radiation, adjuvant cisplatin (40 mg/m<sup>2</sup>) was offered to all patients in maximum six courses. Most patients started the courses of cisplatin; however, some had dose reduction, delay, or discontinuation of use due to toxicity problems. The follow up included clinical examinations every 3<sup>rd</sup> month for the first two years, then twice a year the next three years, and thereafter once a year. If symptoms of recurrent disease were seen, MR imaging of pelvis and retroperitoneum and X-ray of thorax were performed. The time between diagnosis and the first event of relapse (progressive disease) or cancer related death was recorded. The endpoints employed in this study were locoregional control (no relapse within the irradiated pelvic volume including regional lymph nodes), progression free survival (PFS, survival without locoregional and/or distant relapse), and disease specific survival (DSS, not dead from cervical cancer). Patients who died of causes not related to cervical cancer were censored at the time of death.

In paper I and II, we used a uniform cohort of only squamous cell carcinomas, excluding the nineteen adeno- and adenosquamous carcinomas, to investigate the expression and phosphorylation of EGFR (Table 1). This selection of patients was chosen since it has been shown that the prognostic value of individual tumor markers such as EGFR differs with the histological subtype (Lindstrom et al. 2009; Hellberg et al. 2009). In paper I, patients were only included if paraffin section of their tumors were available for IHC, while in paper II, an additional subset of patients were included from which we had gene expression data from Illumina bead arrays.

In paper III, both squamous-, adenosquamous-, and adeno-carcinomas were included (Table 1), since we were investigating more general properties of the tumor, and since the number of squamous cell carcinomas was not great enough to enable statistical significant analyses of the data. Among the 78 tumors on which DCE-MR images had been taken, gene expression data were available for only 46 patients.

## **Tumor specimens**

At the time of diagnosis, one to four biopsies at a size of approximately 5 x 5 x 5 mm, were collected from different locations of the tumor, immediately snap-frozen in liquid nitrogen, and stored at - 80°C. A mixture of multiple biopsies were used for microarray analysis and aCGH,

to minimize confounding effects caused by intratumor heterogeneity in gene expressions and copy numbers (Lyng et al. 2004). For the gene expression analysis, all biopsies had more than 50% tumor cells, as evaluated in hematoxylin and eosin stained sections. For aCGH, this limit was not strictly followed, since it was possible to correct for tumor fraction using GeneCount (Lyng et al. 2008). For 191 patients, a separate biopsy was fixed in neutral 4% buffered formalin and embedded in paraffin for IHC analyses. Samples for gene expression profiling and DNA copy number analysis were available for 155 and 86 patients, respectively. When analyzing hypoxia related parameters, it is extremely important that the biopsies are rapidly frozen, since the living tumor cells otherwise would be influenced by the lack of supply of blood and nutrients, and increased hypoxia or necrosis could occur. This could lead to gene expression responses that are not reflecting the conditions in the tumor *in vivo*. Thus, a strength of our study is the rapid handling of the biopsies after collection from the tumors, as described above.

## Cell cultures

Cell cultures were used to create cervical cancer specific hypoxia gene sets for paper III. Three cervical cell lines were chosen; HeLa, SiHa and CaSki. While SiHa is a squamous cell carcinoma of origin, HeLa cells come from a cervical adenocarcinoma while CaSki has its origin in epidermoid cervical carcinoma (Meissner 1999). Both CaSki and SiHa contain integrated HPV16, while HeLa cells have integrated HPV18 sequences, thus they all carry high risk HPV-types. There are differences between the cell lines when it comes to oxidative stress response (Ding et al. 2007), thus using all three cell lines when investigating hypoxia provided hypoxia gene sets reflecting several of the most common cervical cancer subtypes. Correct identity of cells was ensured by STR profiling, using Powerplex 16 (Promega, Madison, WI). This kit amplifies 15 STR loci and amelogenin for gender identification: Penta E, D18S51, D21S11, TH01, D3S1358, F GA, TPOX, D8S1179, vWA, Amelogenin, Penta D, CSF1PO, D16S539, D7S820, D13S317 and D5S818. The size of the PCR products was determined in a Megabace1000 using the software Fragmentprofiler (GE Healthcare, Chalfont St. Giles, UK).

## Microarray techniques

### aCGH

To determine the gene dosage of EGFR in paper I, BAC-based array comparative genomic hybridization (aCGH) was performed (Fig. 9). While oligonucleotide-based CGH arrays have a better resolution (Ylstra et al. 2006), the BAC-based arrays provide a higher signal to noise ratio, which increases the reliability of the data. To derive absolute DNA copy numbers from the clinical aCGH data, GeneCount, a method previously established in our group (Lyng et al. 2008), was utilized. This method both considers the intratumoral heterogeneity in DNA copy numbers, and also corrects for the normal cell fraction and tumor ploidy. Since cervical cancers are frequently aneuploid and tumor samples often contain considerable amount of normal cells, it is important to correct for these parameters to obtain correct copy number data.

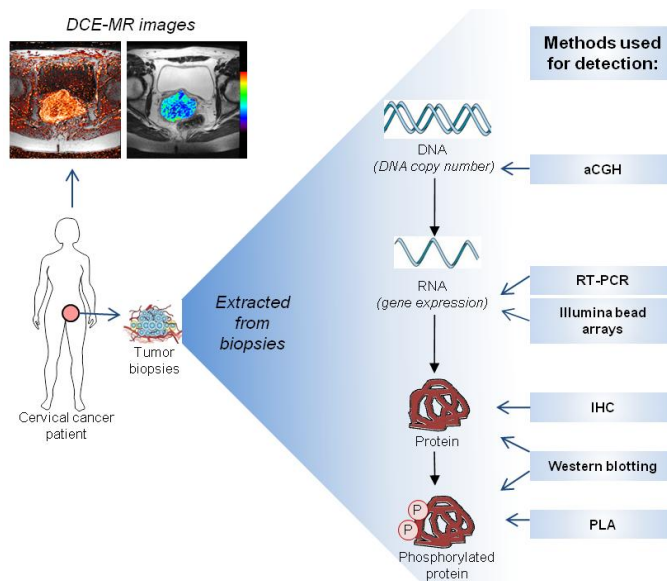


Figure 9: Methodology of the study



## **Gene expression arrays**

Microarray analyses are frequently employed to investigate the gene expression of individual tumors to get a snapshot of what is happening at the molecular level in the tumor cells. One disadvantage of doing transcriptome analyses is that gene expression levels are not necessarily correlated with protein levels. However, current methods to assay genome wide protein expression, such as mass spectrometry, have several major limitations, such as sensitivity and an inadequate dynamic range (Vestal 2011), and are thus presently not acceptable alternatives. Furthermore, gene expression assays do give an indication of what is occurring at the molecular level in the cells, and may therefore be utilized to get insight into the differences that may be present between different tumors at protein level.

In our study, Illumina gene expression bead arrays were chosen for the gene expression analysis in paper I and III (Fig. 9). Regarding choice of microarray platforms, it was demonstrated by the MicroArray Quality Control (MAQC) project that all those which are commercially available have a relatively high level of interplatform concordance (Shi et al. 2006). The Illumina Bead Array was shown to be among the very best performers across various technical measurements, and it includes all genes in the genome and provides separate data for most isoforms. Unfortunately, however, it did not contain information about all the EGFR isoforms mentioned in paper II. Patients were only selected for Illumina gene expression arrays if their tumors contained at least 50 % tumor cells. This strengthens our study since the contribution of gene expression from tumor cells was consequently stronger than from other cells in the microenvironment of the tumor. However, it may have influenced the analysis of the data in that a high tumor cell fraction might indicate a more aggressive tumor, thus patients from which we obtained gene expression data could be expected to have a worse prognosis compared to the rest of the patients. This could theoretically lead to an underestimation of the importance of a factor in relation to survival, since the range of survival parameters would be less than if all patients were included in the gene expression analysis, making it more difficult to reach statistical significance. A concern when performing gene or protein assays on a limited number of biopsies is the possible influence of the intratumor heterogeneity on the assay data. However, a study by Bachtiary et al. (2006) on cervical tumors showed that most genes were in fact

expressed relatively homogeneously within each tumor, with most of the variability occurring in-between tumors from different patients.

### **Downstream analysis of microarray data**

LIMMA (linear models for microarray data) is a method for finding differential expression of data arising from microarray experiments, where the central idea is to fit a linear model to the expression data for each gene (Smyth 2004). The analysis is made stable by borrowing information across genes using Empirical Bayes and other shrinkage methods. To correct for multiple testing, the Benjamin and Hochberg False Discovery Rate (FDR) method is applied. However, we did not take the FDR values into consideration in paper II. By limiting the results with a fixed FDR value, the number of false positives is lowered; however, true positives may also be lost in the process. Since the purpose of using LIMMA in our study was to give clues to whether the ectodomain EGFR tumors had an aggressive phenotype and not to give accurate reports about single genes, we applied a liberal p-value limit of 0.05 to derive a wide window of hypotheses to be explored further.

In paper II, the genes with  $p < 0.01$  from the LIMMA analysis were allowed to generate a network of their known protein interactions, using information from three different interaction databases (Kerrien et al. 2007; Keshava Prasad et al. 2009; Stark et al. 2006). The  $p < 0.01$  gene could create a connection to another gene in the data set if they had a known protein interaction and if the other gene had a  $p < 0.05$  from the LIMMA analysis. All the networks created with more than three members when including first and second degree interactions, were visualized using the Cytoscape software (Shannon et al. 2003). This allowed us to identify genes which could be of importance for the aggressive phenotype of tumors expressing only ectodomain EGFR isoforms in the membrane.

Gene-set methods are used to explore whether certain genes in biological pathways or genes linked via related biological functions (gene-sets) are differentially expressed according to a binary phenotype of interest. Gene Set Enrichment analysis (GSEA) is widely used for this purpose, but there are certain limitations to this method which makes Significance Analysis of Microarray for Gene Sets (SAM-GS) a good alternative, as reviewed in (Dinu et al. 2007). To construct a test statistic, GSEA measures the expression of all the genes to provide a relative

ranking, while SAM-GS requires measurement only of the expression of the genes in the gene set. The SAM-GS approach is preferable since the expression level of other genes should not affect the inference of a single gene set of interest, if this single set is in fact the only variable which is biologically relevant. The use of relative rankings in GSEA also leads to discarding of the information about the degree of association between each gene and the binary phenotype. Additionally, when the biological performance of several gene-set analysis methods was tested, SAM-GS was shown to perform advantageous over other methods such as GSEA (Dinu et al. 2008). In our work, SAM-GS pointed out the importance of hypoxia in tumors with low  $A_{\text{Brix}}$  as measured with DCE-MRI, and encouraged us to investigate further the hypoxic phenotype of these tumors.

## **Protein assay techniques**

### **Immunohistochemistry to investigate protein level**

When investigating protein expression in tumors, the advantage of immunohistochemistry (IHC) is that it allows for the differentiation between normal cells and tumor cells, in contrast to other protein assay methods such as western blotting. Additionally, it may be used to investigate the subcellular localization of proteins, since it enables easy discrimination between membranous, cytoplasmic, and nuclear staining. It is widely used in the clinic both for diagnostic and therapeutic purposes, however, the method has its limitations, and the feasibility of using IHC to evaluate biomarker expression has been greatly debated (Henson 2005; Anagnostou et al. 2010; Hellberg et al. 2009). IHC is generally semi-quantitative and subjective, and the evaluation of a specific protein may vary between studies because of poorly controlled factors such as differences between commercially available antibodies, antibody dilution, antigen retrieval, as well as tissue preparation and fixation, and storage time of the tissue sections (Atkins et al. 2004; Derecskei et al. 2006). Efforts have been made to standardize the IHC assays; however, no guidelines are universally accepted at present (Fetsch and Abati 1999; Anagnostou et al. 2010). It is thus important to carefully choose the proper antibody as well as the dilution, and ensure that the tissue preparation and storage are optimally done. At least parts of the discrepancy between immunohistochemical staining of EGFR and clinical outcome in various cancer types, as well as with the clinical outcome after anti-EGFR

treatment, might result from some of these factors. In our study, IHC with one or two antibodies against EGFR was performed in paper I and II, respectively, to detect the expression of EGFR (Fig. 9). The antibody NCL-EGFR-384 from Novocastra was selected for paper I because of its recognition of the ICD of EGFR, in addition to its ability to recognize nuclear EGFR (Lo et al. 2005), which was recently demonstrated to be a novel prognostic marker in certain cancer types (Lo et al. 2005; Hadzisejdic et al. 2010). The H11 clone from Dako Corporate was chosen as the antibody against the ECD of EGFR in paper II since it is widely used, and it was found to be the only EGFR antibody giving a statistically significant prognostic classification in a study by Anagnostou et al. (2010). For both antibodies, several dilutions were tested, and the one which gave the best distribution of cells between weak and strong staining, was chosen.

### **Proximity ligation assay to investigate the level of phosphorylated protein**

The proximity ligation assay (PLA) was used to detect phosphorylated EGFR in paper I and II (Fig. 9). Using PLA compared to conventional IHC to detect phosphorylated tyrosine kinase (TK) receptors has several advantages (Jarvius et al. 2007). Due to its potent signal amplification, it provides increased sensitivity. More importantly, this method is highly specific, due to the need of the binding of two different antibodies for detection. When using antibodies that are not specific to phosphorylated EGFR, in that they are also detecting other activated members of the ERBB family, one can falsely believe that the results are reflecting phospho-EGFR positive tumors, while it is in fact the subset of activated ERBB family members which is detected. This is crucial when relating these studies to the use of specific ERBB-family inhibitors in targeted treatment of cancer patients. Since it is difficult to make phospho-amino-specific antibodies (Gembitsky et al. 2004; Sun et al. 2001), it may be difficult to achieve a specific result when using common IHC with these antibodies for detection of phosphorylated RTKs. This is where the PLA method comes in useful.

The quality of the primary antibodies used is the main possible limitation to this technique, in terms of efficient binding and recognition of the epitope in question. In our study, both antibodies were tested separately to find the optimal antigen retrieval technique and dilution, and western blotting was performed to ensure binding to the full length EGFR by both antibodies. Additionally, the phospho-specificity of the phospho-EGFR antibody was

confirmed by treatment with a lambda phosphatase, displaying activity towards phosphorylated serine, threonine, and tyrosine residues.

### **Western blotting**

In paper II, western blotting was used to examine the presence of different isoforms of EGFR in cervical cancers (Fig. 9). Western blotting gives a more specific assessment of the presence of the various isoforms compared to IHC, since it allows easy determination of the size of the detected protein, as opposed to the latter method. However, it does not allow distinguishing between normal and cancerous tissue. Additionally, performing western blotting on tumor tissue is challenging and time-consuming, in particular when needing to discriminate the membranous versus cytoplasmic expression of the proteins of interest. Thus, we chose to use this method only on a subset of the patients to confirm that various isoforms of EGFR were indeed expressed in cervical tumors.

### **RT-PCR**

In paper II, RT-PCR was performed to determine the expression of the various alternative transcripts of *EGFR* in cervical tumors (Fig. 9). The primers for the housekeeping gene *GAPDH* was included for all tumors as a positive control, and samples where no band for *GAPDH* were present were considered to be of poor quality and were excluded from the analysis.

### **DCE-MRI**

In contrast to molecular methods, DCE-MRI provides an image of the entire tumor, and will therefore better reflect its heterogeneity. In paper III, DCE-MRI was used to depict the whole tumor (Fig. 9), and a parameter from Brix pharmacokinetic analysis was evaluated in combination with data from gene expression and IHC studies. While the Brix model is commonly used for pharmacokinetic modeling, perhaps an even better known two-compartment model is the one proposed by Tofts, providing the parameter  $k^{\text{Trans}}$ , among others (Tofts 1997). We chose to analyse a Brix model parameter, since the Brix model has some advantages to the Tofts model with regard to the feasibility of use in the clinic. In the Tofts model, the arterial input function (AIF) is needed, and it is technically challenging to perform

accurate and reproducible measurements of the AIF for each patient. The Brix model is simplified so that the AIF is not needed, and it is thus more easily applicable in the clinic (Zwick et al. 2010). However, a disadvantage is that this simplification also may contribute to less precise modeling of the tracer uptake.

When interpreting data from pharmacokinetic analyses, there are various means by which parameter values may be employed to describe the tumor. A common choice is using median or mean values of the various parameters, however, it has been stated that the heterogeneity in tumors is not accurately described using such simple summary statistics (Walker-Samuel et al. 2006). Loncaster et al. (2002) has also suggested that using mean or median values of various parameters to describe the contrast enhancement level may not be the best approach, given the large variations which exists in the tumor. As an alternative, looking at various parts of the distribution of data on a histogram may provide more information, since this approach includes all of the tumor volume. Several studies have shown that using such alternative analysis techniques may better describe tumor heterogeneity, and hence strengthen the use of DCE-MRI in the clinic. In a study by Mayr et al. (2000), the RSI of the 10<sup>th</sup> percentile was a better predictor of tumor recurrence than mean or median signal. In another study, the usefulness of histogram analysis to characterize the heterogeneity of tumors as well as the response to therapy was demonstrated, in that larger standard deviations and heterogeneous distributions of  $A_{\text{Brix}}$  was associated with poor tumor response to treatment (Chang et al. 2004). Furthermore, in a phase I study evaluating the  $\alpha_v$ -integrin receptor antibody (CNTO 95; Centocor), histogram analysis showed significant differences of  $K^{\text{trans}}$  between progressive and stable disease, while no changes in median  $K^{\text{trans}}$  values were seen in response to therapy (Mullamitha et al. 2007). A percentile screening method was therefore previously performed by our group to evaluate DCE-MRI parameter distributions in relation to outcome of cervical cancer patients (Andersen et al. 2011). In the current study, a simplified version of this screening method was applied, where all percentiles of the parameter histograms were evaluated in relation to survival of the patients using log rank survival tests. The resulting p-values were plotted against the percentile values, providing an overview of the prognostic regions of the percentile values, enabling easy identification of the percentile areas with the strongest associations to survival. This method also provides an indication of the robustness of the parameter as a predictive factor, i.e. the size

of the prognostic interval of the parameter. In this regard, large bands of significant percentiles were found for  $A_{\text{Brix}}$ , indicating its robustness. In paper III, the most prognostic region of the percentile values of  $A_{\text{Brix}}$  from this work was chosen for further analysis in combination with gene expression profiling and IHC.

To investigate the relationship between  $A_{\text{Brix}}$  and specific phenotypes such as hypoxia, proliferation, and radiation resistance, specific gene sets for each particular phenotype were included in the analysis. It is suggested that approximately 1.5 % of the human genome is transcriptionally responsive to hypoxia (Denko et al. 2003), and we chose to create hypoxia gene sets specific for cervical cancer for our analysis since it is shown that the transcriptional response to hypoxia differs between tumor types (Chi et al. 2006). Other studies have identified gene sets which are specific for various additional phenotypes, and some of these were chosen to represent the remaining phenotypes of interest in our analysis.

## Discussion

In this thesis, the expression of EGFR at various levels was explored in cervical cancer, and the findings were related to the outcome of the patients to clarify the potential of EGFR as a biomarker. Furthermore, the prognostic value and molecular background of a DCE-MRI parameter was investigated, in order to explore its potential to reflect an aggressive phenotype and thus be used in the implementation of biomarkers in the clinic.

### Novel insight into the expression of EGFR in cervical cancer

In cervical cancer, EGFR is overexpressed at both gene and protein level (Ngan et al. 2001; Yamashita et al. 2009). However, the relationship between the various expression levels and the activation of EGFR in cervical cancer, i.e. between gene dosage, protein, and phosphorylation level of EGFR, is not fully known. This was investigated in our first study, by performing aCGH, IHC and PLA on tumor material from patients with locally advanced cervical cancers. We found that approximately two thirds of the tumors displayed protein expression of EGFR, while about 14 % of the tumors were found to have amplifications of the 7p chromosomal region which harbors the *EGFR* gene. This is consistent with two other studies showing amplification of *EGFR* in 12% and 10% of their cervical tumors (Kersemaekers et al. 1999; Iida et al. 2011). Another study, however, found this gene amplified in 35% of their tumors (Ngan et al. 2001), and this inconsistency could be due to a different composition of the patient cohort with regard to factors such as lymph node status or stage, as compared to the other studies. Since the latter study only investigated the gene dosage of *EGFR* in 35 patients, it is likely that such differences could have influenced the accordance with our results. In our work, there was no correlation between gene dosage and protein level of EGFR, a finding which is supported by previous studies (Kersemaekers et al. 1999; Ngan et al. 2001). However, when looking only at tumors with gain of *EGFR*, the gene dosage was in fact correlated with the EGFR protein level. Thus, it appears that the higher the gene dosage, the more of the protein is produced, while when gene dosage is normal or decreased, the tumor relies on other mechanisms to increase protein level. Accordingly, it is well known that the expression of EGFR protein can be regulated by a variety of mechanisms other than gene amplification, such as through regulation of promoter activity or deregulation at translational and post-translational



levels (Zandi et al. 2007). It is also known that EGFR expression is influenced by the HPV E5 oncoproteins (Kim et al. 2010), which inhibit the degradation of the receptor protein and influence its recycling pattern. Moreover, a particular region of the *EGFR* gene has been shown to act as an enhancer in some cancer cell lines overexpressing this protein (McInerney et al. 2001), emphasizing the many levels of expressional control. Interestingly, we found that the gene dosage of *EGFR* was correlated with increased levels of phosphorylated EGFR, a phenomenon which to our knowledge has not yet been explored in locally advanced cervical cancer. This was not limited to the tumors with gain of *EGFR*, as for the level of EGFR protein, but was valid across all tumors. As proposed in our published paper, it is possible that gain of the *EGFR* gene leads to a more stable overexpression of the protein compared to other regulatory methods, further promoting phosphorylation of EGFR and leading to prolonged receptor activation. Furthermore, in cases where the gene dosage of *EGFR* in the tumor is decreased, the tumor might not be able to induce a sufficiently stable increase in expression of the protein to promote phosphorylation. However, this relationship needs to be explored further in a larger data set, since gain of *EGFR* only occurred in a minor part of our patient group.

### **The role of ectodomain EGFR isoforms in cervical cancer**

In many of the cases without gain of *EGFR* where the protein was highly expressed, little or no phosphorylation of EGFR was found. This could indicate that a high EGFR expression is beneficial for the tumor even when the protein is not phosphorylated and thus not active in driving TK signaling. Recently, it was shown that a TK independent role of EGFR is important for survival of tumor cells, providing one possible explanation for this finding (Weihua et al. 2008). Moreover, there is additional complexity to the story of EGFR in cancer which may be associated with this phenomenon; In addition to the full length 170kD EGFR protein which is composed of the ECD, TD, and ICD, three more isoforms consisting of various parts of only the ECD are expressed in various tissues (Albitar et al. 2010; Reiter and Maihle 2003; Reiter et al. 2001). Since these ectodomain isoforms are lacking the ICD where the TK domain is located, they cannot be phosphorylated and are consequently unable to contribute to the TK signaling pathway. The role and importance of these isoforms in cancer remains to be elucidated, since they have not been given much attention by the multitude of studies that have investigated EGFR with regard to cancer. However, a few studies have shown a possible tumor

suppressive role of some of the isoforms *in vitro* and in gynecological cancers (Baron et al. 1999; Baron et al. 2003; Baron et al. 2009; Basu et al. 1989; Flickinger et al. 1992). To further contribute to the discussion of the importance of EGFR in cervical cancer, we wanted to investigate the relevance of these isoforms, in addition to the more common full length isoform and its phosphorylation status, in relation to survival of cervical cancer patients. We found that expression of the full length isoform was not associated with survival of the patients, and neither was the level of phosphorylated EGFR. However, patients with tumors expressing only the ectodomain EGFR isoforms in the membrane had a significantly worse probability of progression free survival compared to the remaining patients, decreasing from 74% to 27%. Previous studies have shown conflicting results with regard to the relationship between the expression of EGFR and survival in cervical cancer patients, and our study could help explain some of the inconsistency in these studies. If the ectodomain isoforms are more important than the full length isoforms of EGFR for survival, it is natural to imply that the choice of antibodies, i.e. whether they are detecting the ectodomain isoforms or not (Fig. 10), would influence the results.

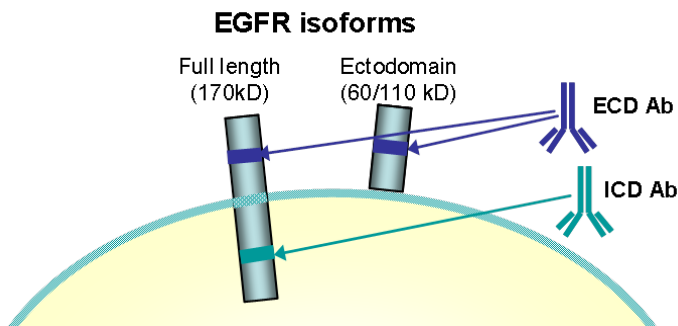


Figure 10: Detection of the various EGFR isoforms with antibodies binding intracellularly or extracellularly, respectively.

The strong prognostic impact of the ectodomain isoforms could give studies using antibodies detecting all isoforms a greater chance of associating EGFR with survival compared to those using antibodies against the ICD. However, when trying to systematically review other studies of EGFR in cervical cancer to see if such a pattern could be found, only few of the studies as well as the antibody manufacturers informed about the binding site of the antibody. Furthermore, the different studies had distinct compositions of the patient cohorts regarding

stage and histological subtypes, and the impact of individual tumor markers may differ with these factors (Lindstrom et al. 2009; Hale et al. 1993; Hellberg et al. 2009). A strength of our study in this regard, was the uniform patient cohort of only squamous cell carcinomas. Furthermore, in the majority of the studies, the importance of EGFR for survival of the patients was not stratified by lymph node status. Since we showed that the ectodomain EGFR isoforms were important only in lymph node negative tumors, this might also have disturbed the interpretation of the results of these studies. Moreover, as discussed in the section “Experimental Conditions”, differences in IHC procedures due to lack of standardization, as well as differences in the quality and specificity of antibody might also explain some of the inconsistency between the results of the various studies.

Our finding of an association between expression of ectodomain EGFR isoforms and an aggressive phenotype may be supported by recent studies showing a role of an ectodomain isoform of ERBB3 (p45-ERBB3) in prostate cancer cell adhesion and metastasis (Vakar-Lopez et al. 2004; Chen et al. 2007). These studies showed that the soluble isoform could bind the plasma membrane of osteoblasts and stimulate them to release factors facilitating invasion and metastasis of the prostate cancer. Another recent study demonstrated a functional link between an ectodomain EGFR isoform and  $\alpha 5$ -integrin in cell adhesion (Wilken et al. 2011), which is an important factor exactly in invasion and metastasis. However, this was related to a tumor suppressive role of this isoform, while we found an association with aggressiveness. The ectodomain EGFR isoforms thus appear to have opposite roles in different cancer types, highlighting the need to achieve a more complete understanding of the functional characteristics of these protein isoforms. Most of the other studies investigating isoforms of EGFR have focused on the 110 kD isoform, while the 60kD isoform seems to be the most prominent in our study. It is thus possible that these isoforms have different functions, explaining the contrasting relations to aggressiveness.

EGFR has been shown to interact with a series of proteins such as E-cadherin, gangliosides, mucins and glucose transporters (Mateus et al. 2007; Miljan et al. 2002; Senapati et al. 2010; Weihua et al. 2008), with some of these interactions only being dependent on the ECD. This implies that the ectodomain isoforms might contribute to aggressiveness through interaction with other proteins, and further investigation of these interactions might be important for

understanding the role of these isoforms. This multitude of possible interaction partners, many of these being membranous proteins, might also explain why the importance of the ectodomain EGFR isoforms in our study was only valid for the membranous staining. Even more obvious interaction partners for these isoforms could be the other members of the ERBB-family, however, results from a study by Ferguson et al. (2000) suggested that domains outside of the ECD are required for heterodimerization of these proteins.

### **The prognostic impact of phosphorylated EGFR in cervical cancer**

The results in paper II showed that phosphorylated EGFR was not important for the survival of the cervical cancer patients in our study. However, we do not want to exclude the possibility of a role of phosphorylated EGFR in cervical cancer overall, because of the following important aspects; Our study population included only squamous cell carcinomas which were locally advanced, and it is thus possible that phosphorylation of EGFR has important functions e.g. for adenocarcinomas or in early stage cervical tumors. Additionally, the full length EGFR with its TK activity could be important in lymph node positive tumors; In our study, we did find a prognostic impact of gain of EGFR which was stronger in lymph node positive tumors (data not presented), which together with the finding of a correlation between gain of EGFR and phosphorylation of the receptor could support this hypothesis. However, this possible differential role of EGFR depending on lymph node status requires further investigation. A study recently demonstrated a correlation between gain of EGFR and poor prognosis in cervical cancer (Iida et al. 2011), but unfortunately, they did not include data regarding lymph node status of their patients. Another important aspect to the evaluation of phosphorylated EGFR is that different sites may be phosphorylated depending on the manner of activation (Filosto et al. 2011). Other sites than the autophosphorylation sites on EGFR are phosphorylated in cancer, such as the SRC-dependent Tyr845, which is shown to be associated with highly aggressive tumors (Boerner et al. 2005). Since we only looked into one of the main autophosphorylation sites, namely the Tyr1068, we cannot exclude that activated EGFR as measured by another phosphorylation site such as Tyr845 is important for the progression of locally advanced cervical cancers. A panel of various phosphorylation sites should thus be assayed in future studies to clarify their role in cervical cancer. Furthermore, another means of investigating the role of phosphorylated EGFR, is through examining the activation status of signaling molecules

downstream of EGFR in the TK signaling network, such as PI3K and MAPK. The expression of EGFR downstream components has only been investigated in cervical cancer by a few studies, and the conclusion of these studies are not clear (Eijssink et al. 2010; Lee et al. 2005).

A limitation of our study is that we did not investigate the importance neither of the other ERBB family members nor the various ligands in relation to EGFR and survival of the patients. The presence of various ligands as well as the choice of dimerization partner for EGFR, have been shown to have implications for the aggressiveness of its TK signaling (Shepard et al. 2008; Tzahar et al. 1996; Olayioye et al. 2000; Humtsoe and Kramer 2010). This is due to the fact that different heterodimers lead to activation of distinct downstream signaling pathways, and because various ligands both contribute to differential phosphorylation of the receptors and influence the choice of dimerization partners for the ERBB-receptors. It is thus possible that full length EGFR could have an important role in some of the tumors if co-expressed with certain other members of the ERBB-family or with a particular ligand. We have previously found that ERBB2, the preferred dimerization partner for EGFR, is expressed in less than 10 % of our patients (unpublished data), limiting its potential role in this context. However, the expression of ERBB3 and ERBB4 could possibly influence the importance of EGFR (Fuchs et al. 2007; Shepard et al. 2008), and it would thus be interesting to look into their co-expression with EGFR in our cervical cancer patients in future studies.

### **Update on the potential of EGFR as a biomarker in cervical cancer**

While EGFR is established as a marker of aggressiveness in various cancer types (Normanno et al. 2006), its potential as a biomarker in cervical cancer is more uncertain. Our findings are not encouraging in this respect, in that we show that no prognostic value is found either in the full length EGFR protein or in its phosphorylation status in cervical cancer patients. However, we revealed that the sparsely explored ectodomain isoforms of EGFR are significantly correlated with poor survival of these patients, highlighting a yet unexplored potential of these isoforms as biomarkers in cervical cancer.

Theoretically, EGFR has seemed like an ideal target for personalized treatment of cancer patients, in that it is overexpressed in many human cancers including cancer of the cervix, and since its relation to aggressiveness was thought to be well established. However, it has proven

difficult to predict which patients will have an effect of agents targeting EGFR, and the response to EGFR targeted therapy has been disappointing in several cancer types. A multitude of possible mechanisms for resistance to these agents have been proposed (Chung et al. 2005). The existence of various isoforms of EGFR in different cancers is one of the factors which has been suggested to influence the response to therapy, in that they may either serve as a sink or as an alternate target for EGFR-directed therapeutics (Wilken et al. 2011). The presence of these isoforms may thus interfere with pharmacokinetic measurements of the protein and/or the therapeutic efficacy. Our results support this opinion, since we show that the measurement of EGFR by IHC is influenced by the presence of various isoforms, which could contribute to a discrepancy between the measured levels of EGFR and response to therapy. However, previous statements on this issue have only considered a tumor suppressive role for the EGFR isoforms, while we suggest an aggressive phenotype of tumors expressing only ectodomain isoforms. Consequently, if these isoforms indeed have aggressive properties in cervical cancer, their presence would not only confuse the measurement of the relative presence of full length EGFR. It could even be hypothesized that treatment with the monoclonal antibody cetuximab could have a better effect in cervical cancer patients compared with treatment with TK inhibitors, since the binding of monoclonal antibodies to the ectodomain is normally followed by internalization of the receptor. This would be particularly interesting to investigate in lymph node negative cervical cancer patients. However, since it is not known if cetuximab indeed will have an impact on these truncated forms of EGFR, this hypothesis needs further exploration.

## **The biological background of a DCE-MRI parameter as a biomarker in cervical cancer**

The current uses of MRI in the clinic are in tumor detection, anatomical characterization, staging and therapy monitoring (Choyke et al. 2003), as discussed in the Introductory section, while DCE-MRI is not yet implemented in the treatment regimes. However, there are several potential areas of use for DCE-MRI, such as predicting response to treatment, and assessing tumor biology through radiogenomics (Padhani and Miles 2010). In this regard, DCE-MRI may provide a non-invasive means of getting insight into the molecular mechanisms that contributes to aggressiveness and treatment resistance in a particular tumor. Various DCE-MRI parameters such as RSI and peak enhancement have been investigated as predictive markers of response to

treatment in cervical tumors (Gong et al. 1999; Hawighorst et al. 1998; Mayr et al. 2000), and in this study, we showed that the  $A_{\text{Brix}}$  parameter from the Brix model have prognostic value in cervical tumors. However, this parameter has not yet been the subject of radiogenomic analysis, in that the molecular background underlying its aggressiveness has not been elucidated. In our study, we aimed to explore this issue by comparing the prognostic DCE-MRI parameter  $A_{\text{Brix}}$  in cervical cancer patients with gene expression data from the same patients. We showed that low levels of  $A_{\text{Brix}}$  were significantly associated with hypoxia, a finding that was validated in an independent patient group.

### ***Imaging of hypoxia as a biomarker in cervical cancer***

While the mean  $O_2$  tension in normal tissue is 50 mmHg (7%  $O_2$ ), tumors may have an  $O_2$  tension as low as 0 mmHg, with a mean of 10 mmHg (1.5%  $O_2$ ) (Hockel and Vaupel 2001). The reason for this frequent hypoxic phenotype of tumors is that the formation of new blood vessels in the tumor often is inadequate, while the newly developed vessels may be aberrant, both leading to inadequate supply of oxygen (Brown and Giaccia 1998; Thomlinson and Gray 1955). Studies have demonstrated that approximately 60% of locally advanced squamous cell carcinomas of the cervix show areas of hypoxia and/or anoxia (Vaupel et al. 2001). Additionally, it has been shown that hypoxic cervical tumors are more resistant to therapy (Lyng et al. 2000), and more likely to metastasize (Chaudary and Hill 2009). It has thus been proposed that tumor hypoxia may be used to predict response to radiotherapy and chemotherapy similarly to when molecular prognostic biomarkers are used for this purpose (Sakata et al. 2006). Using hypoxic areas as a target for biological agents has also been suggested. For these purposes, it is necessary to distinguish hypoxic tumors from oxygenated tumors. The present “gold standard” for detecting oxygenation status of a tumor, is using polarographic measurements of the partial pressures of  $O_2$  within the tumors with micro sensor techniques (Stone et al. 1993). However, measuring hypoxia through this method is invasive, unpleasant for patients, and it is only feasible for accessible tumors. Using non-invasive imaging technologies to determine hypoxia is thus a tempting alternative. In clinical studies, PET measuring the  $^{18}\text{F}$ -fluoromisonidazole ( $^{18}\text{F}$ -FMISO) probe is the most widely used imaging endpoint to depict hypoxia (Vallabhajosula et al. 2011). FMISO-PET has been used in patients with head and neck cancer or non-small cell lung cancer to assess the extent of hypoxia and/or

as an indicator of treatment response. However, PET alone has poor anatomical definition, and needs to be used in conjunction with CT to provide tumor images of high accuracy (Lardinois et al. 2003).

It has previously been suggested that DCE-MRI may be used to depict hypoxia in tumors, as indicated by the correlation between DCE-MRI parameters and tumor  $pO_2$  (Cooper et al. 2000), and by the inverse correlation that was found between DCE-MRI perfusion parameters and hypoxia as measured by FMISO-PET (Jansen et al. 2010). The existence of a relation between hypoxia and various DCE-MRI parameters has also been supported by several recent studies (Yopp et al. 2011; Donaldson et al. 2011; Hagtvet et al. 2011). However, we are the first to demonstrate the relationship between the DCE-MRI parameter  $A_{Brix}$  and hypoxia at the molecular level, and thus to provide detailed information about the hypoxic response depicted by  $A_{Brix}$ . Consequently, our results give additional support to the hypothesis that DCE-MRI may be used to non-invasively select cervical cancer patients with an aggressive hypoxic phenotype.

### ***DCE-MRI in the management of hypoxic tumors***

There have been several attempts in the clinic to overcome tumor hypoxia, but the studies have often been inconclusive (Overgaard and Horsman 1996). Several hypoxia-specific prodrugs have recently been identified, with the most widely tested hypoxic-cell cytotoxin being Tirapazamine (Reddy and Williamson 2009). It has up to the present shown inconsistent results and significant toxicity (Rischin et al. 2010), but is now investigated in an ongoing phase III clinical trial by the Gynecologic Oncology Group (*GOG-219*). Various nitroimidazoles to modify hypoxia have also been extensively studied, but the major limitation is that the most potent drugs are associated with substantial toxicity (Overgaard et al. 1998). Nimorazole is one of the most studied nitroimidazole hypoxia modifiers. It has now been used for several years in combination with radiation treatment of head and neck cancers in Denmark, since it is one of the less toxic nitroimidazole compounds. However, it accordingly has a relatively low efficacy, and is thus not the optimal radio-sensitizer of hypoxic tumors. It thus appears that in order to achieve the desired radio-sensitizing effect of hypoxia modifiers, aggressive treatment is needed, which unfortunately also means higher toxicity. This makes it even more important to be able to identify the relevant hypoxic tumors, to spare patients with relatively oxygenated



tumors from unnecessary treatment and side effects. It has been speculated that developing improved predictive tools for patient stratification could be the most crucial requirement for hypoxia-targeting strategies (Wilson and Hay 2011).

The results of our study have potential therapeutic significance in that they point to the possible use of DCE-MRI to select patients which will not benefit from the current treatment, but who might need alternative or additional therapy to fight the cancer disease. These selected patients might benefit from anti-hypoxia treatment, such as Tirapazamine. It would therefore be interesting to investigate whether  $A_{\text{Brix}}$  could predict response to this or other cytotoxins targeting hypoxia in cervical cancer patients, thus serving as a predictive biomarker for anti-hypoxia treatment. One could also hypothesize that  $A_{\text{Brix}}$  could be further used as a surrogate biomarker for the response of this treatment, in that an increase of  $A_{\text{Brix}}$  during the course of treatment could indicate less hypoxia present in the tumor and thus a therapeutic effect.

Furthermore, the list of hypoxia regulated genes associated with  $A_{\text{Brix}}$  identified in our study, could be useful as molecular biomarkers or therapeutic targets in the patients with an aggressive hypoxic phenotype. A limitation of our study in this regard, however, is that the protein level was not assessed for any of these genes. A continuation of the work could thus be to investigate a panel of these hypoxia-regulated genes at the protein level, preferably accompanied by their activation status, in relation to the level of  $A_{\text{Brix}}$ . The rationale is that the changes in enzyme activity and pathway fluxes are not fully reflected in changes in gene expression, since post-transcriptional processing is important for these pathways (Rodriguez-Enriquez et al. 2011). Thus, by looking at protein levels or at covalent modification of proteins reflecting their activity in relation to DCE-MRI parameters, even more knowledge about the underlying phenotype could be achieved. If some of these 31 genes could be proven to be of importance for survival of cervical cancer patients also on protein level, either individually or combined, DCE-MRI and  $A_{\text{Brix}}$  could be used to predict which patients would benefit from targeted therapy against these proteins. One potential example is STC2, which has already been proposed as a promising molecular target for the treatment of gastric cancer (Yokobori et al. 2010). Moreover, several of these 31 genes are HIF1 $\alpha$  target genes, or are somehow related to HIF-signaling. This may thus indicate that patients with low  $A_{\text{Brix}}$  and thus a high hypoxia score could benefit from therapy

targeting HIF1 $\alpha$ , which is already developed and is being tested in several other cancer types (National Library of Medicine (US) 2011). Furthermore, a few of the genes in the signature are known to be part of another transcriptional program activated by hypoxia, namely the unfolded protein response (UPR). Predicting response to targeting of these or other components of the UPR could thus be another potential area of use for the  $A_{\text{Brix}}$  parameter.

It is important to emphasize that the DCE-MRI hypoxia gene signature not necessarily includes the most important hypoxia genes in cervical cancer overall, but rather the hypoxia genes which are reflected in  $A_{\text{Brix}}$ . Thus, our data only gives an indication of which of the hypoxia inducible genes that are associated with the aggressiveness observed in the low  $A_{\text{Brix}}$  tumors. Furthermore, the signature is based on the genetic response of cervical cancer cells exposed to only a single hypoxic condition, namely 24h in 0.2% O<sub>2</sub>. It is thus not reflecting the various kinds of hypoxia which potentially are present in the tumors, such as acute or cycling hypoxia, not to mention anoxia. Additional genes or pathways regulated under these conditions are thus not included in our signature, but may nonetheless be important for the aggressive phenotype of tumors with low  $A_{\text{Brix}}$ . To get a more complete picture of the hypoxic genotype reflected in  $A_{\text{Brix}}$ , further studies could include more of these conditions, and possibly also take into account the effect of pH which has shown to confuse the interpretation of hypoxia regulation (Sorensen et al. 2005). DCE-MRI predicted signatures based on such studies could provide a list of genes differing from the 31 genes presented in our study, which might or might not reflect more broadly the hypoxic phenotype underlying low  $A_{\text{Brix}}$ .

## Conclusions

It is necessary to identify biomarkers and their signaling pathways to understand the mechanisms behind the development of aggressive cervical cancers and to identify possible targets for therapy. In the present work, EGFR was chosen as our subject of investigation since there currently is a great interest in attempting to treat cervical cancer patients with therapeutics targeting EGFR. However, we believed that more insight into the biology of EGFR was needed to guide the choice of EGFR therapy and to guide the selection of patients to receive treatment. We unraveled new relationships between various levels of EGFR expression in cervical tumors, including the unknown association between gene dosage and level of phosphorylation. Moreover, although our study still leaves behind some unanswered questions, it clearly indicates the importance of understanding which aspects of the complex EGFR story that are relevant for the cervical cancer patients before EGFR is targeted in the clinic. In particular, we point to the importance of considering the presence of ectodomain EGFR isoforms together with lymph node status of the patients when designing future clinical trials using EGFR-directed agents.

Nonetheless, to implement molecules such as EGFR as markers in the clinic for selecting patients for targeted treatment, an applicable method is needed. Great potential lies in the field of imaging as a method to implement biomarkers, in particular when combining them with radiotherapy where MRI already is used for treatment planning, such as for cervical cancer. In the current work, we demonstrated the potential of combining molecular data with imaging parameters and showed how non-invasive DCE-MRI may be utilized to identify patients with hypoxic tumors – a well known biomarker of aggressive disease. Our findings further indicate the possibility of using DCE-MRI during the course of treatment to repeatedly monitor the effect of an eventual hypoxia targeted therapy. Hypothetically, it could also be possible to find imaging parameters that reflect aggressive phenotypes related to the expression of specific molecular biomarkers, such as ectodomain EGFR, encouraging further studies in the area of radiogenomics.

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