Department of Clinical Chemistry
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mRNA-expression-based classification of solid tumors

Development of accurate amplification-based quantification techniques

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ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Medical Faculty of the University of Helsinki in Auditorium 2 of the Biomedicum Helsinki on June 14, 2002, at 12 o'clock noon.

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To Annika, Sam and Oskar

Contents

List of Original Publications

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals:

I Stenman J, Lintula S., Hotakainen K, Vartiainen J, Lehvaslaiho H and Stenman UH. Detection of squamous-cell carcinoma antigen-expressing tumour cells in blood by reverse transcriptase-polymerase chain reaction in cancer of the uterine cervix. Int J Cancer 1997; 74: 75–80.

II a Stenman J, Finne P, Stahls A, Grenman R, Stenman UH, Palotie A and Orpana A. Accurate determination of relative messenger RNA levels by RT-PCR. Nat Biotechnol 1999; 17: 720–2.

II b Stenman J, Finne P, Stahls A, Grenman R, Stenman UH, Palotie A and Orpana A. Supplement to: Accurate determination of relative messenger RNA levels by RT-PCR. Nat Biotechnol 19991; 17: web extra.

III Stenman J, Hedstrom J, Grenman R, Leivo I, Finne P, Palotie A and Orpana A. Relative levels of SCCA2 and SCCA1 mRNA in primary tumors predicts recurrent disease in squamous cell cancer of the head and neck. Int J Cancer 2001; 95: 39–43.

IV Stenman and Orpana A. Accuracy in amplification. Nat Biotechnol 2001; 19:1011–2.

V Stenman J, Lintula S, Rissanen O, Finne P, Hedstrom J, Palotie A and Orpana A. Quantitative detection of low-copynumber messenger RNAs differing at single nucleotide positions. Submitted.

Abbreviations

3SR	self-sustained sequence replication
AMV	avian myeloblastosis virus
ASCUS	squamous cells of undetermined significance
ASO	allele-specific oligonucleotide
ATCC	American type culture collection
cat G -K, -L, and -S	cathepsins G -K, -L, and -S
CDK	cyclin dependent kinase
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
CIN	cervical intraepithelial neoplasia
CPM	counts per minute
CV	coefficient of variation
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
EDTA	ethylene diamine acetic acid
FIGO	international federation of gynecology and obstetrics
FRET	fluorescent resonance energy transfer
HGSIL	high grade squamous intraepithelial lesion
HMC	human mast cell chymase
HPV	human papilloma virus
ISH	in situ hybridization
LCR	ligase chain reaction
LGSIL	low grade squamous intraepithelial lesion
mRNA	messenger ribonucleic acid
NASBA	nucleic acid sequence-based amplification
NK-cell	natural killer cell
nt	nucleotide
Pap	Papanicolaou (-smear / screening)
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pRb	retinoblastoma protein
RCA	rolling circle amplification
RNA	ribonucleic acid
RSL	reactive site loop
RT-PCR	reverse transcriptase-polymerase chain reaction
SAGE	serial analysis of gene expression
SCCA	squamous cell carcinoma antigen
serpin	serine proteinase inhibitor
TAS	transcription-based amplification system
TNM	tumor–node–metastasis
VLP	virus-like particle

Abstract

The aim of this study was to utilize molecular biology techniques to develop novel prognostic methods for the management of solid tumors. Squamous cell carcinomas of the head and neck region and the uterine cervix have served as model diseases for this purpose.

In the early stages of this study we developed a sensitive non-quantitative assay for detecting occult epithelial cells in the circulation of patients with cancer of the uterine cervix, based on detection of squamous cell carcinoma antigen mRNA in peripheral blood. With this technique we were able to detect circulating cells in 6 out of 15 patients and in two pregnant women at term. The low level of target template in the blood samples, however, resulted in uncontrolled variability and we turned the focus to developing accurate techniques for quantification of gene expression in microscopic tissue sections. This led to the development of an RT-PCR based technique for accurate quantification of the relative levels of the highly homologous SCCA1 and SCCA2 mRNAs occurring in biopsy specimens of tumor tissue and normal epithelium.

The relative levels of SCCA1 and SCCA2 mRNA in squamous cell tumors of the head and neck region was demonstrated to correlate with tumor types displaying an aggressive behavior as well as with the recurrence free survival of the patients. The consistent but subtle variations in the relative levels of the SCCA genes were beyond the quantitative accuracy of the most commonly used techniques for mRNA quantification. Our results suggest that the ratio of SCCA2 to SCCA1 mRNA is a potential prognostic molecular marker in head and neck cancer. As the number of patient samples with available follow up data was limited in our study, the clinical applicability of this novel marker remains to be verified.

A mathematical algorithm was developed in order to estimate the degree of variation caused by stochastic factors in techniques striving to quantify molecules in highly diluted solutions. The algorithm was tested empirically using a highly optimized competitive RT-PCR based assay to quantify relative levels of rare mRNAs at different sensitivity levels. We were able to show that sampling variation accounts for a major part of the inaccuracy associated with highly sensitive amplification and detection techniques.

In conclusion, we have been able to show that highly accurate amplification based mRNA quantification can be achieved by comparing the levels of closely related transcripts to each other. This concept was demonstrated using a competitive RT-PCR system with end-point detection. The technique as such is unlikely to be directly applied as a tool for mRNA based grading in the future due to the relatively complicated methodology. The concept of relative quantification of closely related mRNA:s is however readily applicable to a real-time PCR setup as well as to other amplification and detection technologies.

Review of the literature

1. Introduction

In the process of carcinogenesis, accumulation of multiple successive genetic alterations eventually lead to the development of malignant cells lacking normal mechanisms of cellular control. In a healthy individual, a sophisticated surveillance system that repairs potential carcinogenic mutations or alternatively destines affected cells for apoptosis prevents accumulation of successive carcinogenic mutations in the same cell. Mutations that target some part of this surveillance system, thereby partly inactivating it, can however, result in an accelerated rate of uncorrected mutations, and subsequently to the development of cancer [Fearon, 1990]. Such mutations affect one of two major cellular functions, namely the control of cell proliferation or DNA proofreading and repair. In addition there are a vast amount of genes involved in normal physiologic processes that in a malignant environment can supply tumors with beneficial properties. Expression of genes involved in angio- and lymphangiogenesis, cellular adhesion, proteolytic degradation and defense against immunological surveillance provides subsets of malignant cells or cell clones with a selection advantage during the process of carcinogenesis. The accumulation of a pattern of expressed genes that favor the tumors potential for expansion, invasion and metastatic spread eventually leads to an aggressive behavior of the tumor. The expression of such genes and the resulting expression profiles of malignant cells provides the targets for molecular characterization and classification of cancer.

At present, pathological diagnosis and classification of human neoplasia is based on recognition of histological features, with immunophenotyping and other molecular techniques serving as ancillary methods. A combination of pathological classification and clinical criteria are used to distinguish prognostically distinct classes and subclasses of tumors in clinical settings [Alizadeh, 2001]. Due to the variation in the clinical behavior of cancers within the currently accepted classifications, predictions of response to treatment and clinical outcome are in many cases inaccurate on the individual level. The discovery of new molecular markers for classification of malignant disease has been accelerated by the development of DNA microarray technology. Array generated expression profiles have been successfully used to define novel cellular pathways and biologically-distinguishable tumor sub-categories [Alon, 1999; Ross, 2000; Perou, 2000; Bittner, 2000; Hedenfalk, 2001]. The vast amount of expression data produced by microarray techniques enables development of new clinical test for monitoring genes involved in the control of specific cellular functions. Novel mRNA expression based tests are likely to be integrated with already established techniques to enable a novel classification of human malignancies in order to guide specific treatment of individual patients.

We have developed techniques to measure the mRNA expression of the serum tumor marker squamous cell carcinoma antigen genes SCCA1 and SCCA2, and studied the expression of these genes in squamous cell carcinomas of the head and neck region and in the uterine cervix. Both cancer forms evolve from mucosal squamous epithelial cells, but result in widely different disease patterns and outcome. In head and neck cancer a high mortal-

ity rate combined with significant morbidity caused by aggressive treatment protocols generates a need for new predictive techniques that would aid clinicians in determining the optimal treatment strategy for individual patients. The disease pattern of cervical cancer differs significantly from that of head and neck cancer as cytological screening has led to early diagnosis in developed countries and the majority of cases can be cured by radical therapy. The need for prognostic indicators in this type of cancer is restricted to the subgroup of patients in which cellular atypia of undetermined significance is found in cytological screening.

2. Head and neck cancer

2.1 Overview

An estimated 860.000 new cases of head and neck cancer was diagnosed in 1990 [Parkin, 1999] making it the seventh most common form of cancer in the world. Of these new cases 634.000 were diagnosed in men and 228.000 in women. Head and neck cancer includes squamous cell carcinomas arising from the mucous membranes lining the upper aerodigestive tract. This area comprises the lip, the oral and nasal cavities, the paranasal sinuses, the larynx, and pharynx [Clayman, 1996]. (fig. 1) Carcinomas of the salivary glands and nasopharynx are considered distinct etiologic groups of head and neck cancer. Other tumors of this region including skin, brain, ocular and infrequent sarcomas and lymphomas are considered as separate entities [Vokes, 1993]. Patients with stage I or II disease generally undergo either surgery or radiotherapy with curative intent. Over 80% of patients presenting with stage I disease and over 60% of those with stage II will be cured [Vokes, 1993]. Patients presenting with stage III–IV local or regionally advanced disease will typically receive sequential radiotherapy and extensive radical surgery including a neck dissection. Fewer than 40% of these patients achieve 2-year disease free survival and different combinations of chemotherapy with the standard regimens are used in order to achieve organ preservation and prolonged survival in specified settings [Clayman, 1996]. Mortality in head and neck cancer is mainly due to primary treatment failure or to locoregional recurrent disease in patients initially reaching remission [Vokes, 1993].

2.2 Etiology

Squamous cell carcinoma of the head and neck is a heterogeneous disease with distinct patterns of presentation and behavior. The majority of head and neck cancers are associated with heavy consumption of tobacco and alcohol [Vokes, 1993]. Many of the patients present with multiple foci of cancerous or precancerous cells, so-called field cancerization [Slaughter, 1953], throughout the upper aerodigestive tract. Molecular data has provided evidence of the causal role of these substances in the process of head and neck carcinogenesis [Brennan, 1995]. A feature typically affecting this group of patients is second primary tumors which develop at an annual rate of 4–7% of all head and neck cancer patients irrespectively of the result of the primary treatment. These new and potentially fatal cancers typically evolve in the anatomical regions of smoking-related carcinogenesis and in the bladder. Two different types of viral infections have been linked to the etiology of head and neck cancer. More than half of the squamous cell carcinomas arising in the oropharynx contain DNA of human papilloma virus (HPV) [Gillison, 2000]. It is thought that HPV-positive oropharyngeal tumors

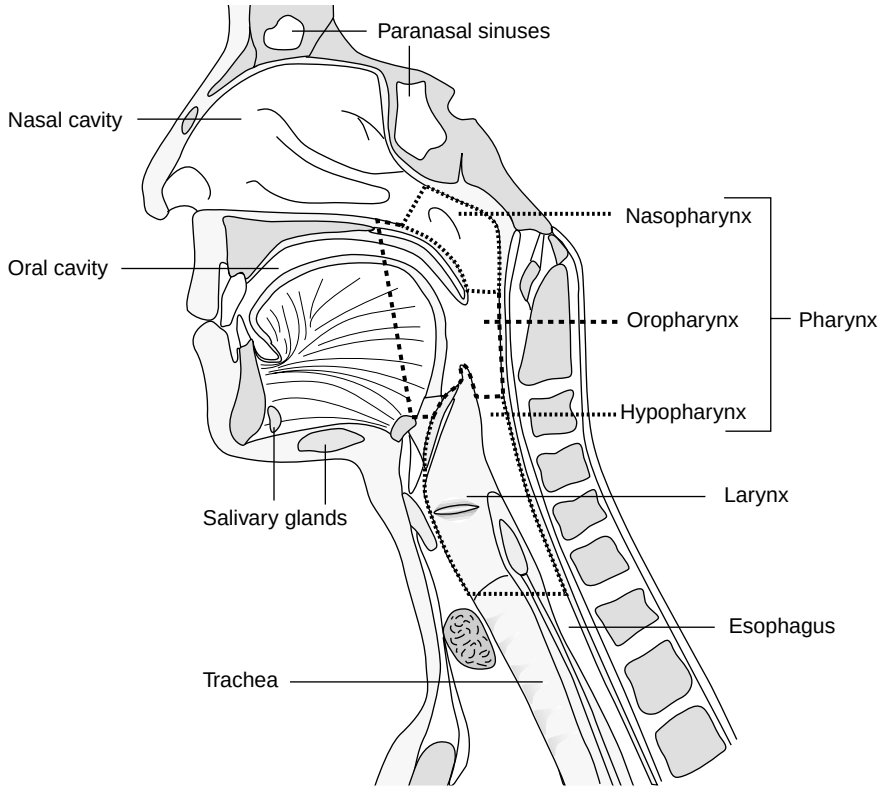

Figure 1. Sagittal section of the upper aerodigestive tract. Squamous cell carcinoma of the head and neck evolves from the epithelium lining the lip, the oral and nasal cavities, the paranasal sinuses, the larynx, and the pharynx. (Adapted from Vokes EE et al. Head and neck cancer. NEJM 1993; 328: 184–94.)

comprise a distinct disease entity initially caused at least in part by an HPV infection. Nasopharyngeal carcinoma represents a distinct entity of head and neck cancers which has been strongly linked to Epstein-Barr virus (EBV) infection [Chien, 2001; Lo, 1999; Shotelersuk, 2000]. This type of head and neck cancer, often presenting with poorly differentiated or undifferentiated histology, is endemic in northern Africa and many Asian populations and differs from squamous cell carcinoma of other sites in the head and neck. In this type of disease the rates of local control are typically high but the development of distant metastases is common and often lethal.

2.3 Molecular basis of oncogenesis

Chromosomal alterations linked to head and neck cancer have suggested specific genetic regions to be involved in the early development of this type of cancer.

The most common genetic change is loss of the chromosomal region 9p21 which is an early event in the progression of these tumors. Deletion of 9p21 results in loss of the Ink4a/ARF locus and subsequently to inactivation of the p16INK4a and ARF proteins. p16 and ARF are both involved in a complex signaling network regulating and interconnecting the activities of RB protein and p53 [Sherr, 2001]. p16 is important in regulating the cell

cycle through inhibition of cyclin dependent kinase (CDK) [Kamb, 1994; Reed, 1996] and keratinocytes in culture have been shown to escape senescence as a result of lost p16 function [Papadimitrakopoulou, 1997; Munro, 1999]. p53 is mutated in more than 50% of all human cancers [Sherr, 2001] and correspondingly approximately half of all head and neck cancers contain a mutation of the p53 gene. The loss of p53 function results in a progression from preinvasive to invasive lesions and increases the likelihood of further genetic progression.[Boyle, 1993; Hartwell, 1994; Koch, 1996]. In about one third of the tumors the oncogene cyclin D1 is amplified resulting in constitutive activation of cell cycle progression. Cyclin D1 amplification has been associated with invasive disease [Berenson, 1989; Callender, 1994; Jares, 1994]. On the contrary no tumor suppressor genes have been directly linked to the chromosomal regions most commonly lost [Nawroz, 1994; Ah-See, 1994].

2.4 Treatment strategies

New treatment strategies have succeeded in improving quality of life through conservation of organ function, such as swallowing and speech as well as minimizing cosmetic handicap. These strategies include surgical techniques for preservation of laryngeal function, selective neck dissection and reconstruction of tissue defects. Hyperfractionation of radiotherapy allowing escalation of the total dose by minimizing damage to normal cells and accelerated fractionation protocols shortening the duration of therapy and thereby reducing the potential for tumor repopulation between fractions, have been tested in phase III clinical trials. These have documented a 10–15% improvement in local control in stage II–IV patients, however, without a significant increase in overall survival [Forastiere, 2001]. Induction chemotherapy followed by radiotherapy has become a standard treatment in advanced cancer of the larynx and has resulted in preservation of a functional larynx in about half of the cases [The Department of Veterans Affairs Laryngeal Study Group, 1991; Lefebvre, 1996]. Concurrent chemotherapy and radiotherapy has been shown to be an effective alternative for selected patients with oropharyngeal, nasopharyngeal and locally advanced unresectable tumors of various origins. The role of adjuvant chemotherapy for high risk patients initially reaching remission is currently under evaluation [Forastiere, 2001]. Promising novel therapies targeted specifically to patients with head and neck cancers include the epidermal growth factor-receptor antagonists CDK inhibitors and replication competent adenoviruses. Monoclonal antibodies against epidermal growth factor receptor, which is overexpressed in over 90% of head and neck cancers, have been shown to inhibit cell proliferation mediated through autocrine and paracrine pathways by blocking ligand binding [Mendelsohn, 1997; Grandis, 1998]. Small molecule drugs based on this finding have reached phase I and II trials. Replication-competent adenoviruses have been developed that selectively replicate in and cause lysis of p53 deficient cells [Heise, 1997; Heise, 1999]. For the time being aggressive surgical resection remains the cornerstone of therapy in head and neck cancer [Forastiere, 2001].

2.5 Pathological classification

Staging of head and neck cancer has changed very little over the passed decade [Forastiere, 2001; Vokes, 1993]. Traditional staging systems including TNM classification determined by clinical examination, imaging procedures and surgical findings, histological grade and clinical patient characteristics only weakly predict the outcome of treated individuals [Forastiere, 2001]. A variety of serum tumor markers including SCCA, CEA, CA 19-9, sialic acid and ferritin have been evaluated for detecting occult malignancy and monitoring treatment of head and neck cancer. None of these have however reached widespread clinical use due to lack of sensitivity and specificity [Rassekh, 1994]. Serial postoperative measurements of squamous cell carcinoma antigen (SCCA) is being used in some

centers for early detection of recurrent disease [Snyderman, 1995; Lara, 1995]. Several other parameters have been proposed including new imaging techniques and identification of sentinel nodes by lymphoscintigraphy [Alex, 2000; Koch, 1998]. Genetic changes in the form of loss of heterozygosity [Rosin, 2000], allelic imbalance [Partridge, 2000] and nuclear DNA ploidy [Sudbo, 2001] have been used to characterize early head and neck cancer lesions. Mutations in the p53 gene have been used to detect residual cancer cells in tissue margins at the periphery of the tumor [Brennan, 1995]. Detection of occult epithelial cells in lymph nodes has been accomplished by detecting p53 mutations [Partridge, 2000] as well as tissue specific expression of MET [Cortesina, 2000] and SCCA [Hamakawa, 1999] genes. Molecular techniques have also been used to determine the relation between a primary tumor and subsequent lesions [Leong, 1998; Califano, 1999] in order to aid clinicians in determining whether to treat a second primary lesion aggressively or to settle for palliative treatment in the case of distant metastatic disease. Despite that many of the new molecular techniques for characterization and classification of head and neck cancer have been shown to provide predictive information on the risk of disease recurrence, none so far have reached widespread clinical practice.

3. Cancer of the uterine cervix

3.1 Overview

Cervical cancer was diagnosed in 371.000 new cases in 1990 [Parkin, 1999]. It is the second most common cancer in women worldwide. Almost 80% of the cases occur in developing countries where cervical cancer accounts for 15% of cancers in women. The incidence and mortality in cervical cancer have decreased substantially in most developed countries due to long-standing Papanicolaou (Pap) screening programs [Parkin, 2001]. Approximately 80% of cervical cancers are squamous cell carcinomas, while 20% are mixed, adenomatous or metastatic to the cervix [DiSaia, 1997]. The most important cause of cervical cancer is human papilloma virus (HPV) infection [zur Hausen, 1976; Boshart, 1984], and HPV DNA can be found in the vast majority of tumors and intraepithelial lesions [Cannistra, 1996]. When detected at an early stage, cervical cancer is largely a curable disease. Microinvasive stage IA1 disease has less than a 1% risk of lymph node spread and can thus be effectively treated by hysterectomy or cervical conization, yielding nearly 100% cure rates [National Institutes of Health Consensus Development Conference statement on cervical cancer., 1997]. Early forms of invasive disease (stage IA2–IIA) are treated by surgery alone or in combination with external beam or intracavitary radiotherapy. Five-year disease free survival rates of 80–90% have been reported for this group of patients. Locally advanced (stage IIB, III and IVA) disease has been mainly treated with radiotherapy resulting in survival rates of 65%, 40% and less than 20% respectively [Cannistra, 1996]. (fig. 2) The combination of chemo- and radiotherapy as a standard regimen for treatment has significantly improved survival rates in these groups [Whitney, 1999; Morris, 1999; Rose, 1999; Keys, 1999; Peters, 2000], however long-term follow up data is still lacking. Adenocarcinoma has a poorer prognosis at every stage compared to squamous cell carcinoma, and the prognosis of adenosquamous cancer is poorer still [Canavan, 2000].

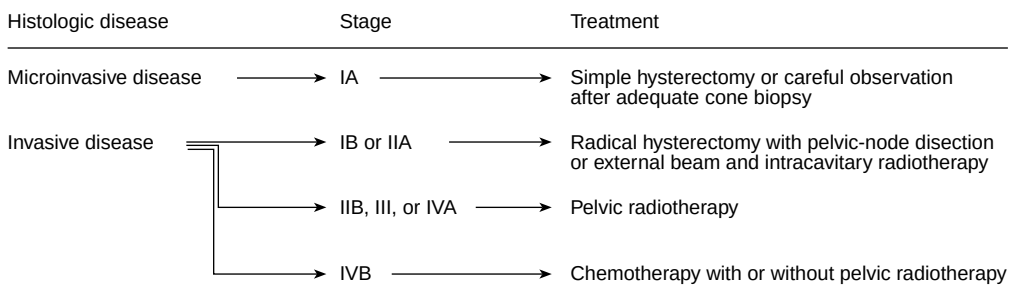

Figure 2. Algorithm for managing micro-invasive or invasive cervical cancer. (Adapted from Cannistra SA and Niloff JM. Cancer of the uterine cervix. NEJM 1996; 334: 1030–8.)

3.2 Etiology

The human papillomavirus (HPV) plays a central role in the etiology of cervical cancers. More than 95% of these cancers have been shown to harbor HPV DNA. [Bosch, 1995; Ngelangel, 1998; Chichareon, 1998; Walboomers, 1999]. HPV is a double-stranded DNA tumor virus ubiquitous in higher vertebrates. Of the more than 100 characterized types of HPV capable of affecting humans at least 25 infect the genital tract [Janicek, 2001]. Low-risk varieties of HPV are associated with viral condyloma or mild dysplastic changes in the cervical epithelium which rarely progress into invasive disease. In contrast high-risk variants of HPV such as types 16, 18, 31, 33 and 35 are often observed in association with moderate to severe dysplasia and carcinoma in situ as well as in invasive cancer [Crum, 1984; Koutsky, 1992; Crum, 1985; Willett, 1989; Howley, 1991]. Other risk factors for cervical cancer include smoking, immunosuppression, young age at first intercourse, high number of sexual partners, high parity and low socioeconomic status [Cannistra, 1996; Janicek, 2001]. Of these risk factors most are linked to sexual behavior and an increased risk of acquiring HPV. Smoking has consistently been shown to be an independent risk factor and even passive smoking has been associated with an increased risk of specifically squamous cell carcinomas, but not with adenocarcinomas or adenosquamous carcinomas [Brinton, 1986; Slattery, 1989]. HIV infection is associated with a higher incidence of cervical dysplasia, more multifocal lesions, more rapid progression and higher recurrence rates when compared with HIV-negative women [Maiman, 1993; Klein, 1994]. HIV-positive women with invasive cancer also tend to present with advanced stage disease, have resistance to therapy and a shortened survival [Rellihan, 1990; Maiman, 1993]. There has however been no consistently documented rise in the incidence of invasive cervical carcinomas in HIV-infected women [Janicek, 2001].

3.3 Molecular basis of oncogenesis

The molecular basis for oncogenesis in the majority of cervical cancers is explained by the function of viral oncogenes E6 and E7 which are integral structures of the HPV-family of viruses [Howley, 1991; Scheffner, 1991]. The gene product of E6 has been shown to induce degradation of resident p53 protein by binding to it [Werness, 1990; Scheffner, 1990]. Inactivation of p53 through either E6 binding or mutation is a central component in the process of malignant transformation in cervical cancer, and p53 has been shown to be mutated in the minority of cervical cancers not infected with HPV [Scheffner, 1991; Park, 1994]. E7 targets another closely related tumor suppressor gene product, the retinoblastoma protein (pRb) by binding to it and changing its phosphorylation state [Munger,

1989; Dyson, 1989]. Phosphorylation of pRb disrupts its ability to suppress E2F transcription factor and cell cycle progression into S-phase is accelerated [Sherr, 2001]. HPV types are stratified into, low- intermediate- and high-risk categories based on their association with high grade and invasive cervical lesions [Syrjanen, 1988; Lorincz, 1992]. The high-risk HPV types exhibit greater inactivation of p53 and RB and a single amino acid difference has been shown to be critical for the different binding affinities for RB [Heck, 1992]. Certain loci of the human leukocyte antigens (HLA) or major histocompatibility antigens has been associated with specific types of HPV in cervical cancers [Ellis, 1995; Allen, 1996]. Interactions of HPV with specific histocompatibility antigens might explain the differences in patterns of progression to invasive disease in patients infected with the same HPV type [Janicek, 2001].

3.4 Screening and treatment strategies

The widespread screening programs based on Pap-smear cytology have reduced the incidence and mortality from cervical cancer by up to 60–80% in some developed countries [Miller, 2000]. The age limits for initiation and discontinuation of screening as well as the screening frequency varies somewhat in different countries [Sawaya, 2001]. New screening techniques include liquid-based cytology collection and analysis [Payne, 2000]. Computerized analysis of Pap-smears [Colgan, 1995; Ashfaq, 1995; Brown, 1999] and HPV testing [ALTS Group, 2000; Schiffman, 2000; Solomon, 2001]. Ablative treatment for microinvasive and early invasive forms of cervical cancer is associated with excellent results and has changed little over the last decades. Combination of chemotherapy to radiotherapy and surgery in locally advanced and high-risk early stage disease seems to bring about an increase in overall survival in these patient groups. Novel preventive treatment strategies such as development of vaccines against the most prevalent high-risk HPV types could theoretically prevent more than 300.000 cervical cancer cases per year worldwide [zur Hausen, 2001]. Presently a number of pharmaceutical companies as well as research laboratories are engaged in preclinical and clinical trials of vaccines against high-risk HPV strains. Most approaches are based on virus-like particles (VLP) representing structural proteins L1 or L1 and L2 of papillomaviruses [Zhou, 1991; Zhou, 1992; Zhang, 2000]. The first published report after conclusion of phase I immunogenicity and safety trials on a HPV16 L1 VLP vaccine [Harro, 2001; zur Hausen, 2001] has shown very promising results being highly immunogenic and well tolerated.

3.5 Pathological classification

The Bethesda classification is the most widely used cytologic classification of Pap-smears [Shingleton, 1995; 1989; Kurman; 1991]. Abnormal findings are categorized into low grade squamous intraepithelial lesions (LGSIL), high grade intraepithelial lesions (HGSIL) and squamous cells of undetermined significance (ASCUS). Corresponding classifications also exist for glandular lesions. When cytologic abnormalities are reported, colposcopy is performed to permit visualization and biopsy of the lesion. Biopsy findings are classified as cervical intraepithelial neoplasia (CIN) I, II or III corresponding to mild, moderate and severe dysplasia or carcinoma in situ.The staging system for cervical cancer is based on clinical criteria according to the international federation of gynecology and obstetrics staging system (FIGO) [Creasman, 1995]. Serum marker squamous cell carcinoma antigen (SCCA) has been widely used for monitoring patients treated for advanced cervical squamous cell cancer for early detection of relapses [Brioschi, 1991] and HPV typing of cytology specimen has been shown to provide additional prognostic information to samples classified as ASCUS [Manos, 1999; Solomon, 2001; Hildesheim, 2001]. These groups of patients would benefit from additional prognostic parameters to alleviate determination of optimal follow up and treatment strategies.

4. Squamous cell carcinoma antigen

4.1 Overview

Squamous cell carcinoma antigen (SCCA) is the major component of the TA-4 antigen complex originally isolated from metastatic cervical squamous cell carcinoma tissue [Kato, 1977]. The 14 isoforms of the protein ranging from 42 to 48 kDa were initially grouped into a neutral (pI 6.3–6.6) and an acidic (pI 5.9–6.2) fraction on the basis of their isoelectric points [Kato, 1984]. The neutral and acidic isoforms of SCCA have been shown to be coded by separate genes and have been named SCCA1 and SCCA2 respectively [Schneider, 1995]. Both forms of SCCA are expressed in normal and malignant squamous epithelium [Crombach, 1989; Cataltepe, 2000] but the increased serum levels of SCCA observed in cancer patients is thought to be mainly attributed to increased levels of SCCA2 [Kato, 1984]. The commercially available immunoassay for SCCA (IMx SCC kit, Abbott Laboratories Inc., Abbott Park, IL) does not discriminate between the SCCA isoforms but has reached widespread clinical use as a serum marker in the management of squamous cell carcinoma of the uterine cervix and to some extent in head and neck cancer. The detection limit of this microparticle enzyme immunoassay (MEIA) based method is 0.3 μg/l and the upper reference limit for healthy subjects is 2.5 μg/l. It has been mainly used for monitoring response to therapy and for early detection of relapses [Fuith, 1988; Senekjian, 1987]. The pretreatment levels of SCCA have been shown to correlate mainly with tumor size [Brioschi, 1991] and the prognostic value of pretreatment measurements is being debated [Duk, 1996; Bolger, 1997].

4.2 Genes and proteins

The SCCA1 and SCCA2 genes are tandemly arrayed on chromosome 18q21.3 [Schneider, 1995; Kuwano, 1995]. (fig. 3) The SCCA genes belong to the ovalbumin family of serine proteinase inhibitors (serpins) [Suminami, 1991] and they are flanked by two other closely related serpins, plasminogen activator inhibitor type 2 (PAI2) and maspin. The exon sequences of the SCCA genes display 98% homology, whereas the predicted amino acid sequences are 92% homologous. Recombinant SCCA2 functions as a classical serpin inhibitor of chymotrypsin-like proteinases human neutrophil cathepsin G (cat G) and human mast cell chymase (HMC) [Schick, 1997]. SCCA1 on the other hand, inhibits papain-like cysteine proteinase such as cathepsin K, L and S but not common serine proteinases [Schick, 1998]. (table 1) The majority of amino acids that differ between SCCA1 and SCCA2 reside in the exposed portion of the reactive site loop (RSL). Site-directed mutagenesis directed at either the hinge or the variable region of the RSL abolished its inhibitory activity and it has been suggested that it is the altered sequence and flexibility of the RSL itself that enables cross-class inhibition of cysteine proteinases by SCCA1 [Schick, 1998].

4.3 Expression and distribution

RNA transcripts of SCCA1 and SCCA2 have been detected in human lung, tonsil, thymus, lymph node placenta, prostate and testis using RT-PCR [Cataltepe, 2000]. Using immunohistochemistry with discriminatory monoclonal antibodies for the SCCA1 and SCCA2 isoforms [Cataltepe, 2000] these proteins were shown to be co-localized to the skin, esophagus, tonsil, tongue, thymus, trachea, bronchi, bronchiole, vagina and uterine cervix [Cataltepe, 2000]. Non-discriminatory in situ hybridization experiments have localized SCCA mRNA to the basal and parabasal layers of normal squa-

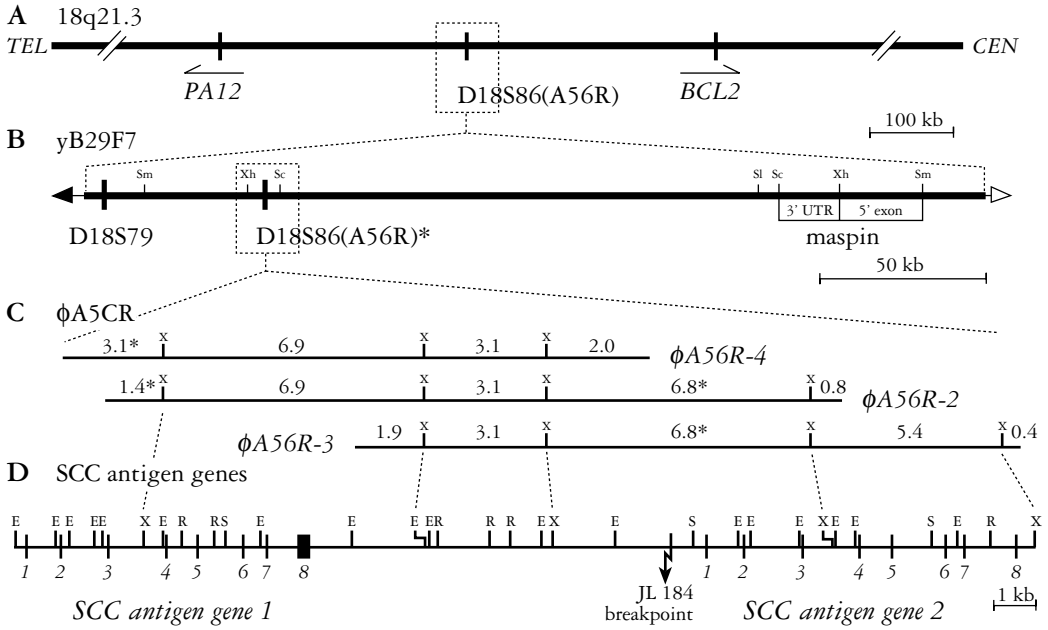

Figure 3. Genomic map of SCCA1 and SCCA2 genes. (Adapted from Schneider SS et al. A serine proteinase inhibitor locus at 18q21.3 contains a tandem duplication of the human squamous cell carcinoma antigen gene. Proc Natl Acad Sci USA 1995; 92: 3147–51.)

Table 1. Inhibitory profiles of SCCA1 and SCCA2

	SCCA1			SCCA2		
Proteinase (final concentration)	SCCA1 (μM)	Ratio (I/E)	Inhibition (%)	SCCA2 (μM)	Ratio (I/E)	Inhibition (%)
catG (42nM)	0.48	11	0	0.4	10	100
HMC (50 nM)	0.48	10	0	0.4	8	100
Chymotrypsin (400 nM)	33.0	83	0	22.0	55	0
Granzyme B (130 nM)	20.0	154	0	13.0	100	0
HNE (330 nM)	33.0	100	0	22.0	67	0
Plasmin (17 nM)	0.48	28	0	0.4	24	0
Proteinase 3 (35 nM)	0.48	14	0	0.4	11	79
PSA (140 nM)	18.0	129	0	14.0	100	0
Thrombin (19 nM)	2.0	105	0	2.0	53	0
Trypsin (450 nM)	33.0	73	0	22.0	49	0
u-PA (100 nM)	2.0	20	0	2.0	20	0
catB (23 nM)	2.0	87	0	2.0	87	0
catS (19 nM)	1.0	100	99	ND	ND	ND
catL (4.3 nM)	0.086	20	93	0.043	10	0
catK (50 nM)	0.7	14	75	0.2	4	0
Papain (207 nM)	0.36	2	91	ND	ND	ND

Inhibitory profiles of SCCA1 and SCCA2. (Modified from Schick C et al. Cross-class inhibition of the cystein proteinases cathepsins K, L, and S by the serpin squamous cell carcinoma antigen 1: A kinetic analysis. Biochemistry 1998; 37: 5258–66, and Schick C et al. Squamous cell carcinoma antigen 2 is a novel serpin that inhibits the chymotrypsin-like proteinases cathepsin G and mast cell chymase. J Biol Chem 1997; 272: 1849–55.)

mous epithelium, in dysplasia and in invasive squamous cell carcinoma [Takeshima, 1992]. Although SCCA1 and SCCA2 are detected in the cytoplasm of normal squamous epithelial cells, neither serpin is detected normally in serum. Expression of SCCA-green fluorescent fusion protein and confocal microscopy has been used to study the release of SCCA1 and SCCA2 from squamous carcinoma cells. In this study the SCCA-fusion proteins were found exclusively in the cytosol and were not associated with nuclei, mitochondria, lysosomes, microtubules, actin or the Golgi. Neither non-stimulated nor stimulated squamous carcinoma cells secreted SCCA into the medium suggesting that the major site of SCCA1 and SCCA2 inhibitory activity is within the cytosol and that the elevated SCCA serum levels of patients with squamous-cell carcinomas may be due to passive release into the circulation [Uemura, 2000].

4.4 Pathophysiological role

The possible role of the serine proteinase inhibitors SCCA1 and SCCA2 in the development of squamous epithelial malignancies is currently unknown. SCCA2 has been suggested to protect the epithelia of the skin and lung against inflammatory degradation by inhibiting leukocyte proteinases HMC and cat G [Schick, 1997]. This theory is strengthened by the earlier finding that inflammatory skin disorders such as psoriasis and atopic dermatitis are associated with increased serum levels of SCCA [Duk, 1989; Campbell, 1990]. The role of the observed cross-class inhibition of cystein proteinases by SCCA1 has not been clarified. It has been proposed that SCCA1 is involved in the control of normal and pathological proteolytic events by inhibition of lysosomal enzymes catK, -L, and -S, released from damaged epithelial cells [Schick, 1998]. Tumour cells transduced with the SCCA1 cDNA have been shown to be more resistant to induced apoptosis as compared to control cells in vitro. These cells also formed larger tumors than corresponding controls in nude mice [Suminami, 2000]. Recombinant SCCA1 has been shown to inhibit migration of NK-cells in vitro. This inhibitory effect is lost by introducing a single amino acid mutation to the reactive site loop [Suminami, 2001]. Suppression of both SCCA proteins using an antisense SCCA mRNA construct has resulted in inhibition of tumor growth and increased infiltration of natural killer cells in vitro which may contribute to the escape of squamous cell carcinoma cells from the host immune system [Suminami, 2001].

5. Techniques for measurement of mRNA-expression

5.1 Strategies for quantification of mRNA expression in malignant cells

In order to characterize the expression of genes critical for the behavior of tumors, pathologists have traditionally relied on immunohistochemistry in paraffin-embedded tissues. Proteins being the effector molecules in most cellular mechanisms, are a natural target for endpoint evaluation of gene expression, and there is no doubt that this will remain a central technique in grading of tumors in the foreseeable future. There are however, several factors limiting the applicability of immunologic techniques for specific purposes. Secretion of proteins to extracellular compartments, complexing by binding to inhibitors or carrier molecules, and highly variable degradation rates render quantitative analysis of gene expression on the protein level relatively

inaccurate. In addition, synthesis of discriminatory antibodies for closely related proteins while retaining sufficient binding affinity, can in some cases be difficult or maybe even impossible. mRNA in contrast, is synthesized, functions and is degraded intracellularly, and can in this aspect be considered a potential target for quantitative analyses of gene expression. Several different techniques enable discrimination of mRNA transcripts on the basis of single nucleotide differences allowing independent analysis of highly homologous transcripts. Measurement of cellular content of specific mRNA transcripts thus provides a versatile method for accurate quantitative analysis of gene expression levels.

Clinical tumor tissue samples generally consist of mixtures of cell types including tumor cells, several different cell types of the normal surrounding tissue, inflammatory cells as well as necrotic cells often in the center of the tumor. In situ detection of mRNA enables visualization of localized gene expression in a heterogeneous surrounding without purification of malignant cells. Detection of multiple transcripts, however, is limited in an in situ format and low-abundance mRNA species often require amplification procedures commonly performed in homogeneous assays. To enable measurement of tumor-derived mRNA from heterogeneous samples in a homogeneous assay, tumor cells can be either physically separated from the surrounding cells or alternatively the analysis can be focused on tissue-specifically expressed genes. Microdissection procedures [Kubo, 1995; Emmert-Buck, 1996; Bonner, 1997; Simone, 1998] have been successfully used to clean tissue samples from other than tumor tissues and to specifically select tumor cells from a heterogeneous surrounding in a tissue sample. With laser assisted microdissection and RT-PCR, amplified mRNA transcripts have been detected even in single cells [Schutze, 1998]. Various immunologic techniques have been utilized in an analogous manner to select tumor cells or to disselect leukocytes in blood and other body fluid samples. A method of circumventing the need for purification of tumor cells is to detect mRNA transcripts displaying tissue- or cell-type-specific expression. This method was originally described for detecting occult malignant cells in blood [Smith, 1991] and analogous techniques have later been applied to tissue samples. Co-variation in the expression of cell-type specific genes has been used to normalize expression profiles obtained from clinical tumor samples with cDNA arrays [Ross, 2000].

5.2 mRNA detection techniques

5.2.1 Hybridization on solid supports and gene expression profiling

The first widely used technique for quantification of specific mRNAs was the northern blot hybridization assay [Alwine, 1977], a modification of the southern blot earlier described for detecting specific sequences among DNA fragments separated by gel electrophoresis [Southern, 1975]. Northern blotting involves size-fractionation of total RNA or mRNA samples and transfer to a nylon or nitrocellulose membrane where specific sequences can be detected by hybridization with typically a ^{32}P-labelled DNA or oligonucleotide probe. In RNA dot blot hybridizations [Kafatos, 1979] the size-fractionation step is omitted and the target RNA is blotted directly onto a membrane. While these early RNA detection techniques allow simultaneous detection of a specific transcript in several samples with labelled probes, the development of reverse dot blot assays enabled detection of multiple sequence targets in single labelled samples [Bugawan, 1991]. Subsequent automation and miniaturization of the dot blot increased the number of genes that could be studied in a single experiment, as specific cDNAs were arrayed onto membranes [Lennon, 1991].

Microarray or chip technology utilizing glass as the solid support has dramatically increased the number of genes that can be studied simultaneously. In a typical experiment mRNA isolated from a tissue sample or a cell line is reverse transcribed and labelled to generate a target that can be detected upon

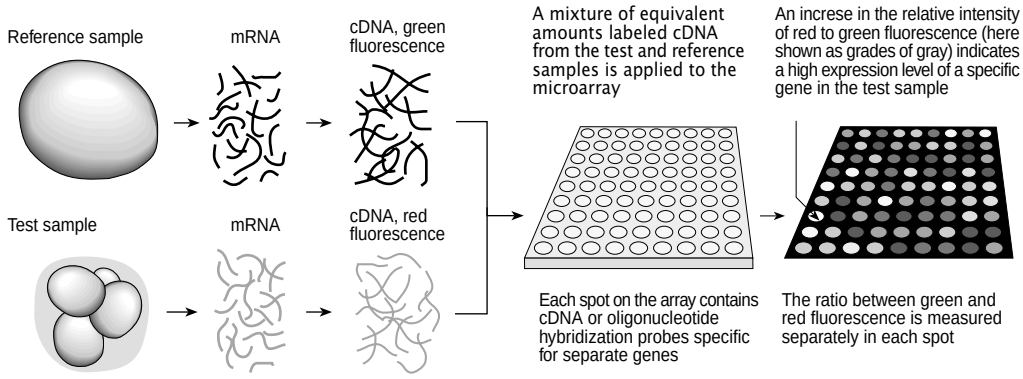

Figure 4. Gene expression analysis using cDNA microarrays. The total pool of mRNA from a test sample and a reference sample is used to prepare fluorescently labelled cDNA by reverse transcription with fluorescently labelled nucleotides. The two differently labelled cDNAs are mixed and hybridized on a cDNA microarray. Each gene to study is represented as a distinct spot of DNA in the array. The relative abundance of specific transcripts in the samples is reflected as the ratio of fluorescence measured in the respective spot. (Adapted from Brown PO and Botstein D. Exploring the new world of the genome with DNA microarrays. Nature Genetics 1999; 21, suppl.: 33–7.)

hybridization to the microarray. Labelling of the target can be done by incorporating labeled nucleotides during reverse transcription, but many protocols require an amplification step in order to produce sufficient amounts of target template for the hybridization reaction. High density oligonucleotide arrays developed at Affymetrix Inc. are manufactured by in situ photolithographic synthesis of oligonucleotide probes based on database sequence information. Hybridization events on the array are monitored by laser confocal fluorescent scanning in up to one million locations. [Fodor, 1991; Southern, 1992; Fodor, 1993; Pease, 1994]. Multiple independent probes specific for different regions of the same target template are used in order to increase signal-to-noise ratio and enable quantification of up to 40.000 target templates. The quantitative accuracy of commercially available oligonucleotide arrays allows discrimination of two-fold differences in expression levels [Lipshutz, 1999]. cDNA microarrays [Schena, 1995; Shalon, 1996; Schena, 1996] are constructed by printing DNA probes on the solid support using ink-jet technology. Two-color fluorescence hybridization is used to quantitatively analyse the relative expression levels of maximally tens of thousands of genes in a test sample in relation to those of a reference sample (fig. 4). By measuring the ratio of differently labelled targets in a mixture, inaccuracy due to variable hybridization kinetics is minimized. The ink-jet technology has been expanded to printing of oligonucleotides which allows synthesis of probes based on database sequence information. Generation of this type of DNA microarrays in an academic environment provides a cost effective alternative for gene expression profiling whereas ready-to-use cDNA microarrays are also commercially available.

An alternative method for analysis of gene expression patterns is Serial Analysis of Gene Expression (SAGE). [Velculescu, 1995] In this technique mRNA is isolated from the source and reverse transcribed. The cDNA molecules are restriction endonuclease digested (anchoring enzyme), ligated to one of two primer specific linker sequences and reduced to 9 nt sequence tags by a second cleavage with a type IIS restriction nuclease (tagging enzyme). The

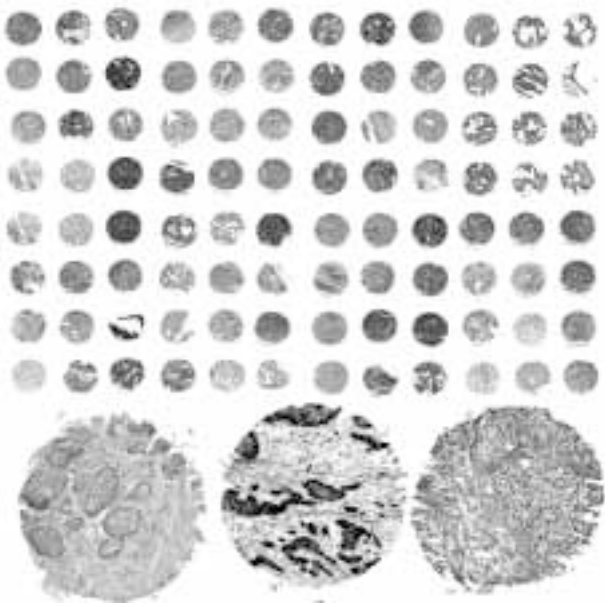

Figure 5. A small region of a tissue microarray (96 spots). Each tissue spot has a diameter of 0.6 mm. Bottom: Higher magnification images of three HE-stained specimens. (Adapted from Kallioniemi O-P. Biochip technologies in cancer research. Ann Med 2001; 33: 142–7.)

tagging enzyme recognizes a specific site on the linker sequences and cuts 9 nt downstream into the tag sequence. The two resulting populations of blunt ended linker-tag sequences are ligated to produce ditags that are subsequently PCR amplified with primers specific for the linker sequences situated in each end. Following amplification the ditags are cleaved from the linker sequences with the anchoring enzyme and concatenated by ligation to form a continuous sequence of sequence tags. Sequencing of the concatenated sequence tags will reveal the identity and the relative frequency of the mRNA transcripts in the original sample. SAGE is a fast method for quantitatively analyzing mRNA expression patterns. The experiments can in principle be performed during a single day and do not require any out-of-the-ordinary laboratory equipment.

In order to characterize new potential markers evolving from gene expression screening experiments on a large number of samples, a technique of generating tissue microarrays on microscopic slides has been developed [Kononen, 1998]. (fig. 5) In this technique up to 1000 cylinders of 0.6 mm diameter are punched from individual "donor" paraffin-embedded tumor blocks and arrayed into a new 45 x 20 mm paraffin block. The height of the cylinders are approximately 3–4 mm allowing up to two hundred consecutive sections to be cut from the block. Classical immunohistochemistry [Bubendorf, 1999] and fluorescent in situ hybridization (FISH) [Schraml, 1999; Richter, 2000] have been successfully used to evaluate the clinical applicability of new candidate marker molecules, whereas mRNA in situ hybridization requiring conservation of intact mRNA in the arrayed sections has been less well documented. A modification of the original protocol for generation of tissue microarrays [Fejzo, 2001] enables generation of arrays from frozen tissue in order to enhance qualitative and quantitative properties of problematic immunohistochemistry applications and especially mRNA ISH assays.

5.2.2 Homogeneous hybridization assays

Detection of single nucleotide differences with allele-specific oligonucleotide probes (ASO probes) was described in 1979 [Wallace, 1979] and used to detect a mutation in the beta-globin gene causing sickle-cell anemia in 1983 [Conner, 1983]. Hybridization of target specific oligonucleotides have been used in a vast number of nucleic acid detection systems but are inheritedly restricted by a certain degree of cross-reaction when used for quantitative detection of single nucleotide differences.

Molecular beacon technology [Tyagi, 1996] combines the simplicity of direct hybridization with an enhanced capacity for discrimination between single nucleotide differences. Molecular beacons are oligonucleotide probes with a stem-and-loop structure consisting of a central target-complementary region that is flanked by two 5–7 nt arm sequences which are unrelated to the target sequence but complementary to each other (fig. 6). The stem is formed by annealing of the two arm sequences. A fluorescent moiety is coupled to one of the arms of the probe and a non-fluorescent quenching moiety to the other. When the probe is in the closed state, the the fluorophore and the quencher are in close apposition rendering the fluorophore unable to fluoresce. When the probe encounters a target nucleic acid, the stem opens due to the greater binding energy of the target complementary central loop sequence to the target, as compared to the binding energy of the self-annealing arm sequences. This forces the fluorophore and the quencher apart from each other and allows the probe to fluoresce upon illumination. Due to the conformation constraint of the molecular beacon probes, a greater specificity can be achieved than with conventional ASO probes [Bonnet, 1999]. Molecular beacons have been successfully used in real-time PCR applications for detection of single nucleotide substitutions causing drug resistance [Piatek, 1998] and multiplex detection of pathogenic retroviruses [Vet, 1999]. Molecular beacons have also been used

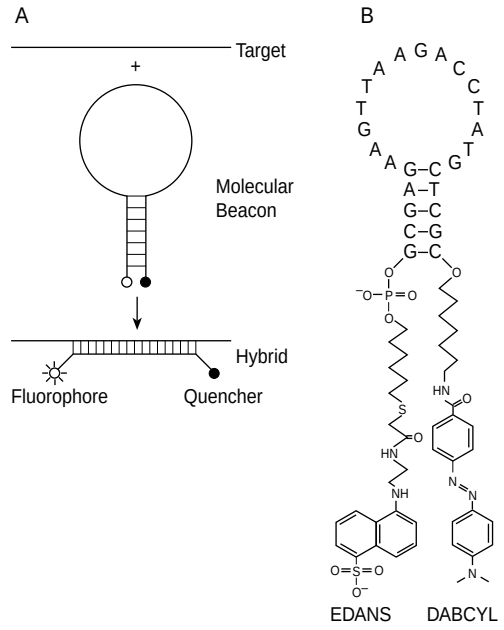

Figure 6. The principle of operation (A) and structure (B) of molecular beacons. The self-complementary hairpin stem of the molecular beacon keeps the fluorophore (EDANS) and the quencher (DABCYL) in close apposition in the absence of target template. When the molecular beacon hybridizes to a specific target the hairpin structure is forced to open up, causing the probe to fluoresce as the fluorophore is released from the quencher. (Adapted from Tyagi, S and Kramer FR. Molecular Beacons: Probes that fluoresce upon hybridization. Nature Biotechnology 1996; 14: 303–8.)

to detect specific mRNAs in living cells without prior amplification [Sokol, 1998]. Fluorescent resonance energy transfer has been utilized to create molecular beacons that are brighter and simplify multiplexing as different beacons can be excited with a common monochromatic light source [Tyagi, 2000].

5.2.3 Enzyme dependent detection assays

Allele-specific ligation [Landegren, 1988] utilizes the enzyme specificity of DNA ligase

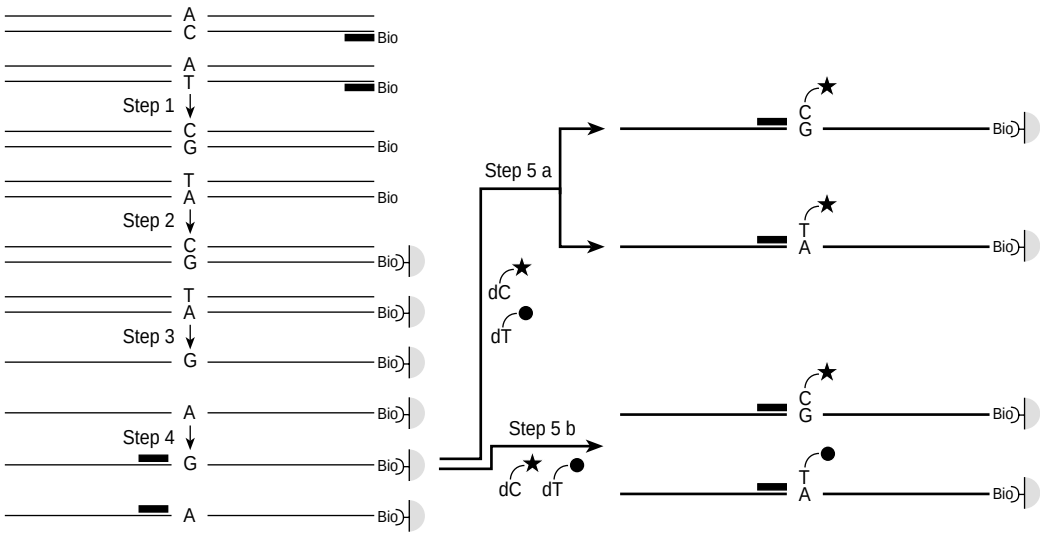

Figure 7. The minisequencing principle. 1. Biotinylation during amplification using a labeled primer. 2. Affinity-capture. 3. Washing and denaturation of the double-stranded PCR amplicons. 4. Annealing of the minisequencing primer. 5a and 5b separate detection of PCR products by minisequencing primer extension with single labeled nucleotides. (Adapted from Syvanen A-C et al. A primer-guided nucleotide incorporation assay in the genotyping of apolipoprotein E. Genomics 1990; 8: 684–92.)

to covalently join two sequence-specific oligonucleotide probes adjacently annealed on a target DNA sequence. This technique was the first model for utilizing enzyme-specificity to detect single nucleotide substitutions. This technology has led to the development of padlock probes [Nilsson, 1994; Nilsson, 1997]. Padlock probes are linear oligonucleotides of approximately 90 nt (80–200) length with a target specific sequence of approximately 20 nt in each end. The padlock probes are circularized by annealing the template specific 3'- and 5'-regions adjacently on a target template and ligating their outer ends. Since DNA and RNA are helical molecules, the formed duplex is coiled at the annealing site one complete turn per 10 base pairs. Circularization of the padlock probe prevents unwinding of the helical structure, and the probe will thus be catenated to the target DNA despite denaturation of the specific part of the probe from the target sequence.

Minisequencing (single nucleotide primer extension) has become the most widely used technique for single nucleotide detection (fig. 7). This technique utilizes the specificity of a DNA polymerase (or alternatively a reverse transcriptase) to incorporate a single nucleotide at the 3'-end of a sequence-specific primer [Syvanen, 1990]. Minisequencing is typically performed in solid phase assays to detect point mutations in amplified PCR products [Syvanen, 1992; Syvanen, 1992; Singer-Sam, 1992]. In such applications, the PCR amplicons are labelled using a biotinylated primer in the amplification reaction. Following amplification, the PCR product is captured on a streptavidin coated solid phase and quantitatively detected by incorporating labelled nucleotides to a target specific primer annealed immediately adjacently to the polymorphic site. Tritium has been commonly used as a detection label in combination with scintil-

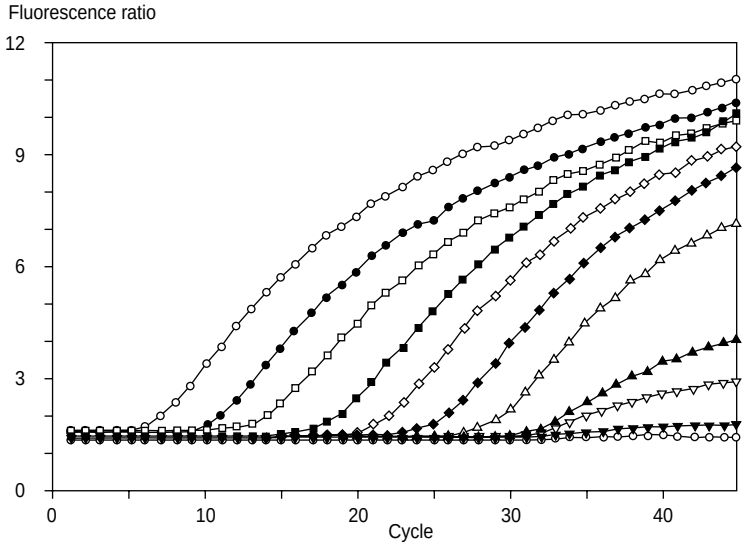

Figure 8. Ratio of fluorescein to rhodamine fluorescence of a dual-labelled hydrolysis probe, monitored at each cycle during a kinetic PCR amplification of 10-fold dilutions of the target template. Threshold cycle values are typically calculated by determining the point at which the fluorescence exceeds ten times the standard deviation of the baseline. (Adapted from Wittwer CT et al. Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques 1997; 22: 130–8.)

lating microtiter plates [Ihalainen, 1994]. Multiplexing of minisequencing reactions has been achieved using fluorescent labels [Pastinen, 1996; Pastinen, 1997] and a homogenous assay format has been described utilizing fluorescent resonance energy transfer (FRET) between a donor fluorophore at the 5'-end of the primer and and acceptor fluorophores coupled to dideoxy terminator nucleotides [Chen, 1997; Chen, 1997]. Minisequencing has been used for quantification of rare mRNA transcripts [Ikonen, 1992; Singer-Sam, 1992] and as a detection method on microarrays [Pastinen, 1998; Pastinen, 2000]. A number of commercial products have been developed based on the minisequencing technology, the most common application being SNP detection.

5.3 Amplification techniques

5.3.1 Competitive RT-PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) has been the mainstream method of mRNA amplification since the advent of PCR in 1986 [Mullis, 1986; Mullis, 1987; Saiki, 1986]. The first quantitative RT-PCR protocols relied on coamplification of an internal standard consisting of in vitro-generated DNA or RNA with the wild type templates [Wang, 1989; Becker-Andre, 1989; Gilliland, 1990]. In this type of assay the initial copynumber of the wild type template is estimated from the ratio of endpoint wild type to internal standard PCR product following amplification. Several different methods of end-point detection of PCR products have been described [Ikonen, 1992; Pannetier, 1993; Alard, 1993]. In an early application of this technique Piatak and colleagues quantitatively determined the viral load of HIV-infected patients [Piatak, 1993]. When carefully optimized to minimize differences in reaction kinetics between the competing templates in the reaction and excluding any possible sources of nonspecific amplification, accurate quantitation over a dynamic range of 6 orders of magnitude can be achieved with this type of technique [Jalava, 1993].

5.3.2 Kinetic RT-PCR

Kinetic or real PCR technology enables fluorometric monitoring of the accumulating PCR products during cycling. (fig. 8) In its simplest form accumulating fluorescence from an intercalating dye is measured [Higuchi, 1992]. Discrimination between coamplified products can be achieved by melting curve analysis provided that the amplified products differ in length. Drawbacks of this technique is that any nonspecific amplification will contribute to the accumulation of fluorescence and thus decrease sensitivity and undermine the quantitative accuracy. Detection technologies based on product identification by hybridization enable increased quantitative accuracy and also allows the use of internal standards in a competitive fashion. In the commercially available TaqMan assay® (PE Biosystems) quantification is obtained by monitoring decrease in fluorescein quenching by rhodamine following exonuclease cleavage of dual-labelled probes that have hybridized to the accumulating PCR amplicons [Holland, 1991; Livak, 1995; Gibson, 1996]. A commonly used commercial assay for Lightcycler® instrument (Roche) is based on fluorescent energy transfer (FRET) of CY5 to fluorescein between probes that anneal adjacently on the accumulating PCR products [Wittwer, 1997]. Alternative detection principles based on FRET include molecular beacons [Tyagi, 1996], energy transfer primers [Nazarenko, 1997] and dye-labeled oligonucleotide ligation [Chen, 1998]. Quantitation in real time assay systems is done from data points gathered during the exponential phase of amplification. Several commercial systems use extrapolation of a threshold cycle at which the accumulating fluorescence from the PCR product exceeds the baseline. The original amount of template in the sample is mathematically derived from the threshold cycle. The kinetic PCR systems offer the convenience of quantitation during cycling which simplifies assay procedures and minimizes the risk of sample contamination. They do not however eliminate the need of an appropriate internal standard if accurate quantitation is to be achieved.

5.3.3 In situ PCR

In situ PCR of DNA targets was first described in 1990 [Haase, 1990]. In situ RT-PCR [Nuovo, 1992] enables sensitive detection of rare mRNA transcripts within cells and tissues as well as co-localization of mRNA transcripts and proteins when combined with immunohistochemistry [Matsuki, 1992; Gosztonyi, 1994]. Technical problems specific for in situ PCR include loss of localization due to diffusion of amplification products as well as primer oligomerization and incorporation of labeled nucleotides into background DNA [Nuovo, 2001]. Several detection methods such as exonuclease cleavage of dual-labeled probes [Holland, 1991], molecular beacons [Tyagi, 1996] and energy transfer primers [Nazarenko, 1997] that allow monitoring of the accumulating PCR amplicons during cycling, are directly applicable to In situ RT-PCR. A technique based on universal energy-transfer labelled primers has been described [Nuovo, 1999] significantly simplifying the experimental procedures of In situ PCR.

5.3.4 Isothermal RNA amplification

Isothermal RNA amplification (Self-Sustained Sequence Replication, 3SR, Transcription-based Amplification System, TAS or Nucleic Acid Sequence-Based Amplification, NASBA) involves a combination of three enzymes to generate a logarithmic amplification of an RNA template without temperature cycling. (fig. 9) In the initial report [Kwoh, 1989], the original RNA template is reverse transcribed using AMV reverse transcriptase and a primer with a T7 RNA polymerase promoter sequence at the 5'-end to generate a (–) cDNA strand. The RNA-DNA hybrid is heat-denaturated and a second primer complementary to a region at the 3'-end of the cDNA initiates

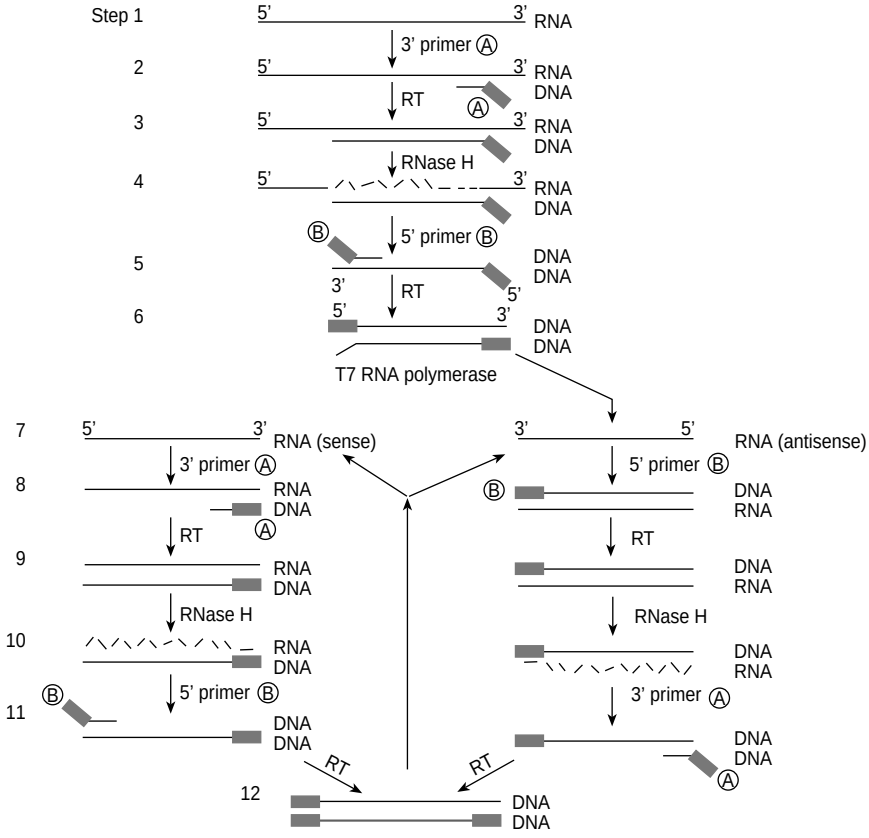

Figure 9. The principle of isothermal RNA amplification (Self Sustained Sequence Replication 3SR, Nucleic Acid Sequence-Based Amplification, NASBA). Amplification proceeds through a continuous cycle of reverse transcription and transcription reactions to replicate an RNA target by means of cDNA intermediates. The generation of double stranded DNA targets for the T7 RNA polymerase (5) is dependent on RNase digestion of the RNA in the intermediate RNA-DNA hybrids. (Adapted from Guatelli, JC et al. Isothermal, in vitro amplification of nucleic acids by a multi-enzyme reaction modeled after retroviral replication. Proc Nat Acad Sci USA 1990; 87: 1874–8.)

reverse transcription of a second DNA strand, thus producing a double stranded DNA template with a T7-polymerase promoter region. Subsequently the T7 RNA polymerase replicates a large number of RNA transcripts from the double stranded DNA template, resulting in a 2–5 x 10^6-fold increase in the copy number of the original target sequence after 4 cycles. In a modification of this technique RNase H was added to the reaction resulting in degradation of the RNA in the RNA-DNA hybrids as the first reverse transcription reaction progresses [Guatelli, 1990; Compton, 1991]. This improved procedure enabled isothermal amplification at 41° C for approximately 90 minutes. Molecular beacons [Tyagi, 1996] have been used to enable real-time detection during isothermal RNA amplification [Leone, 1998].

5.3.5 Probe amplification techniques

Ligase chain reaction (LCR) [Wu, 1989; Barany, 1991] utilizes two probe pairs analogous the sequence of the two respective strands

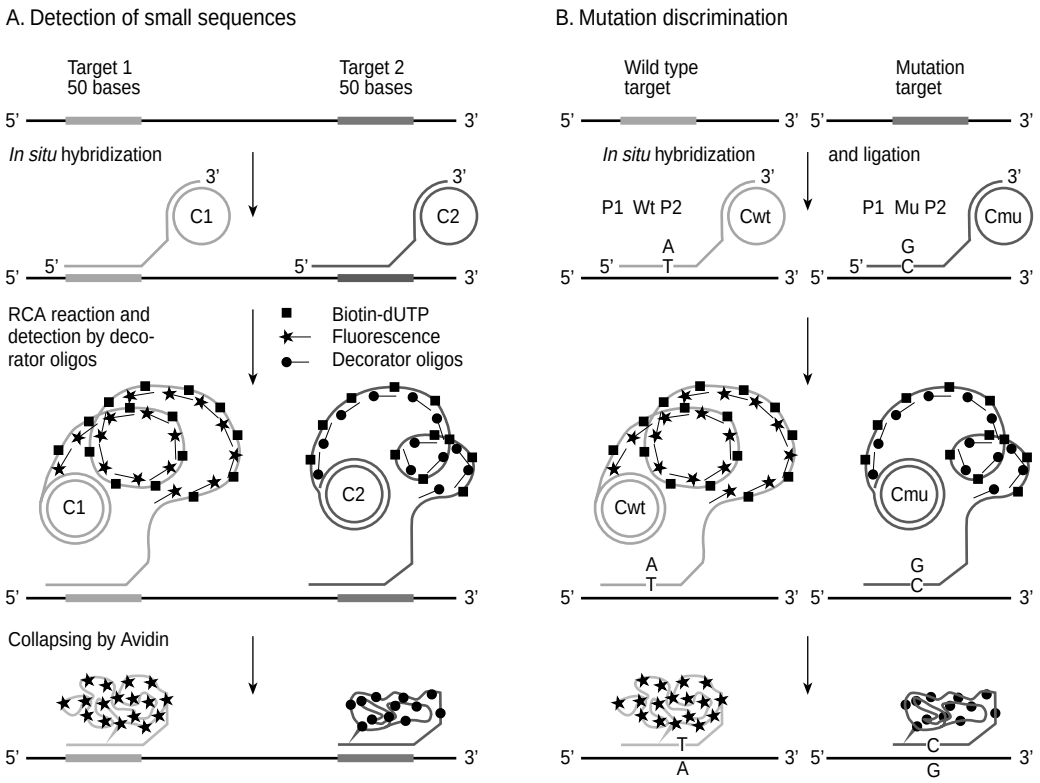

Figure 10. Schematic for detecting small target sequences (A) or point mutations (B) using rolling circle amplification. (Adapted from Zhong, XB et al. Visualization of oligonucleotide probes and point mutations in interphase nuclei and DNA fibers using rolling circle DNA amplification. Proc Nat Acad Sci USA 2001; 98: 3940–5.)

of the target nucleic acid. The reaction proceeds exponentially as adjacently annealed probes are enzymatically ligated and form a template for a pair of complementary probes. The method possesses the enzyme specificity of allele-specific ligation. This relatively simple amplification technique has been used for DNA amplification in numerous genotyping and clinical applications. Rolling circle amplification (RCA) is a probe amplification technique in which a circular DNA 34 nt or longer acts as template for amplification [Fire, 1995; Lizardi, 1998]. (fig. 10) When a DNA polymerase with strand displacement capacity is utilized, the reaction proceeds isothermally creating a single linear complementary DNA strand consisting of multiple tandem repeats of the of the template sequence. Numerous modifications to the RCA protocol have been published. Perhaps the most potential properties of this amplification technique is the possibility to generate localized amplification products for in situ detection of DNA [Zhong, 2001] and mRNA [Zhou, 2001].

Aims of the present study

The aim of this study was to utilize molecular biology techniques to develop novel prognostic methods for the management of solid tumors. Squamous cell carcinomas of the head and neck region and the uterine cervix have served as model diseases for this purpose. The specific aims of this study were:

1) To develop a method for detecting occult malignant cells in the circulation of patients with cancer of the uterine cervix, based on detection of squamous cell carcinoma antigen mRNA in peripheral blood.

2) To develop a method to enable accurate quantification of the relative levels of two highly homologous mRNAs occurring in a clinical sample.

3) To determine the prognostic value of accurately measuring the ratio of SCCA2 to SCCA1 mRNA in tumor tissue of primary squamous cell carcinomas of the head and neck.

4) To mathematically estimate the degree of variation caused by stochastic factors in techniques striving to quantify the absolute and relative levels of molecules in highly diluted solutions.

5) To empirically test the accuracy of a highly optimized competitive RT-PCR based assay to quantify relative levels of highly homologous mRNAs, in relation to the mathematically estimated optimal accuracy at low levels of target template molecules.

Materials and Methods

1. Patients and samples

1.1 Blood samples

Single 3 ml blood samples were drawn from patients under follow up for squamous cell carcinoma of the uterine cervix and from control subjects (I) Two to five 10 ml blood samples were drawn from cervical cancer patients, head and neck cancer patients as well as from corresponding control subject undergoing invasive therapy, before during and after treatment (V). All blood samples were drawn in Vacutainer EDTA tubes (Becton Dickinson, Rutherford, NJ). In order to avoid contamination from epithelial cells from the skin, at least one tube was drawn for other purposes before collection of the sample analyzed in RT-PCR.

1.2 Tissue samples

Grossly prepared tissue samples from three patients treated for squamous cell carcinoma of the uterine cervix was used as positive controls for detection of SCCA mRNA in peripheral blood (I). In order to study the relative mRNA expression of the SCCA1 and SCCA2 genes in malignant and normal tissue we analyzed banked tumor tissue specimens from 30 patients with primary and 11 patients with recurrent squamous cell head and neck tumors and 19 normal mucosal epithelium specimens from the head and neck region of 16 patients surgically treated for various conditions. The condition of the sample and the proportions of different cell types present were determined from a toluidine-blue stained microscopic frozen section and five adjacently located 10 μm sections were transferred to a reaction tube for extraction of total RNA (II, III). Tissue samples had been stored at –80°C (I) and at –40°C (II,III) until analysis. The total amount of tumor tissue samples was limited by the availability of banked specimens from different patients (II, III).

1.3 Cultured cells

Epidermoid carcinoma cell line A431 (ATCC, Rockville, MD) were cultured and used for cell spiking experiments in order to determine the detection limit of the RT-PCR assay (I) and to evaluate the quantitative accuracy of the RT-PCR assay at low template concentrations (V).

1.4 Patients and follow-up characteristics

In order to study the presence of occult epithelial cells in the circulation of cervical cancer patients we studied samples from 15 patients under follow up after treatment for cancer of the uterine cervix and 24 controls (I). To determine the prognostic value of measuring the mRNA ratio of SCCA1 and SCCA2 in head and neck cancer we studied tumor tissue specimens from 30 patients with primary squamous cell tumors and 11 patients with recurrent tumors (II, III). In study V the original aim was to study the possible release of tumor cells into circulation during various forms of invasive treatment. 385 blood samples were obtained from cervical cancer patients undergoing intracavitary radiation therapy, from head and neck cancer patients undergoing radical surgery and from corresponding control subject undergoing surgery

to the head and neck region for other causes. All of the patients included in the studies had been treated with curative intent and informed consent was obtained from all patients and controls. The studies have been approved by the ethical committees of the Dept. of Gynecology and Obstetrics (I, V) and the Dept. of Otorhinolaryngology at the Helsinki University Central Hospital (V) and the Joint Ethical Committee of the Turku University and the Turku University Central Hospital (II, III).

2. Detection and quantification of SCCA mRNA transcripts

2.1 RNA extraction procedures

Total RNA was extracted from microscopic tissue sections by transferring five adjacent 10 µm frozen sections from each sample directly into a chaotropic Lysis buffer (Qiagen, Hilden, Germany) and centrifuged through Qiashredder spin columns (Qiagen) to shear high molecular weight DNA. Total RNA was extracted from the lysates by centrifugation through RNeasy spin columns (Qiagen) where RNA adsorbs to a silica membrane under chaotropic conditions. Prior to elution in diethylpyrocarbonate (DEPC) treated water the RNA extracts were DNA digested in the column membranes according to the manufacturers instructions (II, III).

mRNA was extracted from peripheral blood samples according to two different protocols. In the early stages of the study (I) blood samples were hemolyzed in a hypotonic solution consisting of 1.5 volumes of DEPC-treated water for 5 min. Leukocytes were pelleted and transferred to a 4.0 M guanidium thiocyanate based chaotropic buffer [Chomczynski, 1987] and lysed by vortexing. mRNA was directly captured from the 2:1 diluted lysate using biotinylated oligo-dT probes and captured on streptavidin coated paramagnetic particles (Promega, Madison, WI). At a later stage (V) hemolysis was done by hypotonic shock by 10:1 dilution of blood samples in cold DEPC-treated water and neutralization after 30 sec with 0.1 vol of 10 x PBS. The pelleted leukocytes were lysed in 5.0 M guanidium thiocyanate based buffer, centrifuged through Qiashredder spin columns (Qiagen) and total RNA was extracted using RNeasy spin columns (Qiagen). mRNA was subsequently oligo-dT captured from the total RNA eluate on the wall of 0.2 ml streptavidin coated PCR tubes (Roche diagnostics, Basel, Switzerland).

2.2 Primer design, verification of PCR products and end-point-detection

To optimize stringency of the PCR (II, III, V), all primers were designed to have a 5' or central domain binding to the template stronger than the 3' pentamer. (fig. 11) This was done to ensure that elongation would pursue only after annealing of the entire length of the primer [Rychlik, 1995]. The formation of primer dimers was eliminated by designing all of the PCR primers so that any possible primer-primer duplexes with protruding 5' end enabling formation of amplified dimers possessed considerably weaker binding energies than the 3'-pentamers of the primer to template duplexes. Oligo 5.0 primer analysis software (National Biosciences, Plymouth, MN) was used as an aid in designing and analyzing PCR primers. In order to gain enough freedom to design primers conferring to the above criteria, we did not in any way

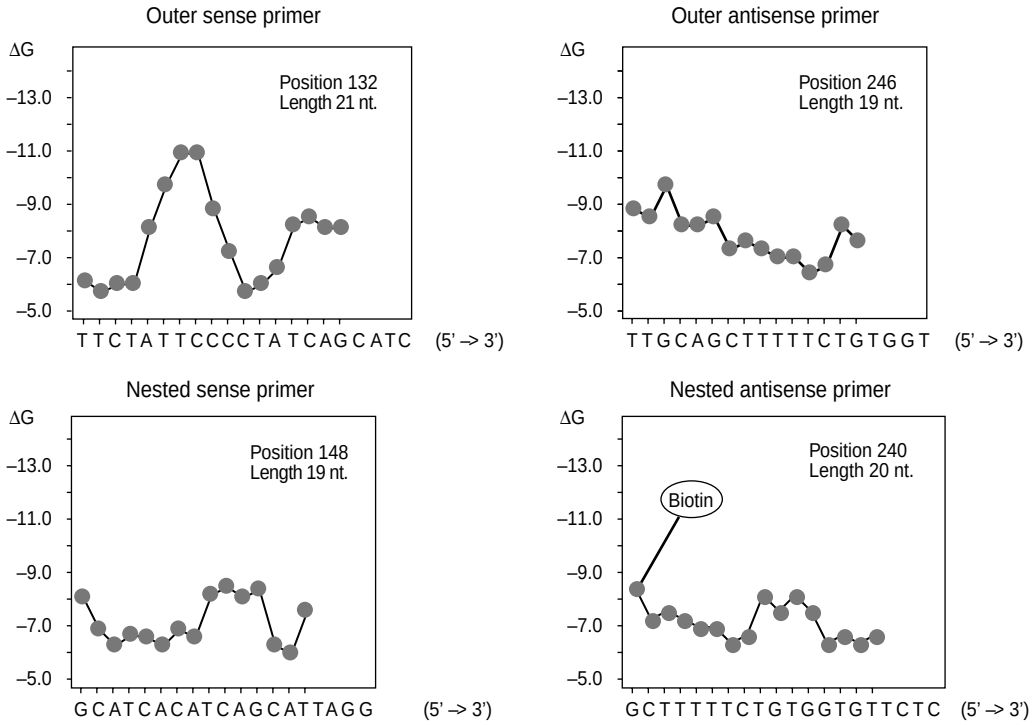

Figure 11. Sequences and internal stability of the PCR primers used for co-amplification of the SCCA1, SCCA2 and internal standard templates. Binding energy was calculated by the nearest neighbor method using Oligo 5.0 software. $\Delta G = \Delta H - T\Delta S$. ΔH = change in enthalpy, T = temperature (25° C), ΔS = change in entropy.

restrict the formation of primer-primer duplexes with protruding 3' overhangs that are unable to amplify.The PCR amplicon spans the exon 2-3 junction of the SCCA1 and SCCA2 genes. The minisequencing primers both bind symmetrically on the exon 2-3 junction. The competitive effect imposed by coamplification of genomic DNA was minimized by DNase digestion of the total RNA extracted from the samples (II, III, V) and by further capturing of mRNA from total RNA extracts (V). PCR products were verified by cycle sequencing on an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA) (I, II) and Southern blot analysis using a ^{32}P-labelled 21-mer oligonucleotide probe (I). A nested PCR protocol was used for all applications. PCR products were endpoint-detected by agarose gel electrophoresis and ethidium bromide staining (I) and quantified in a beta counter by solid phase minisequencing using ^{3}H-labelled nucleotides and 96-well scintillating microtiter plates [Ihalainen, 1994] (II, III, V).

2.3 Internal standard construction

The SCCA1 and SCCA2 templates differ within the PCR amplicon at positions 206, 211 and 212 of the SCCA2 cDNA sequence (Genebank, accession number: U19557). An internal standard was constructed by generating an additional A to T mismatch at position 206 of the SCCA2 cDNA using modi-

fied primers and PCR (II). The generated sequence was amplified with the outer PCR primers, cloned into the pCR® II vector (Invitrogen, Carlsbad, CA) and sequenced from both ends on an ABI 310 genetic analyzer (Applied Biosystems) using the ABI Prism dRhodamine terminator cycle sequencing kit. One thousand (II, III) or twenty (V) copies of the non-linearized vector containing the modified SCCA2 sequence was included in the PCR reactions as an internal standard.

2.4 Measurement of SCCA protein

SCCA protein was measured by immunoassay on an IMx analyzer (Abbott, Abbott Park, IL) using the IMx SCC kit from the manufacturer (I).

3. Statistical analyses

3.1 Evaluation of the SCCA mRNA ratio assay

The Mann-Whitney U-test was used to analyze the statistical significance of the differences between tumor groups and normal epithelium (III). Survival curves were plotted according to the Kaplan-Meier method. The logrank test was used to calculate the statistical significance of the difference in recurrence-free survival between groups and to evaluate the possible confounding effect of commonly used prognostic factors and different cell types present in the samples. The relative risk of recurrence was calculated and the confidence interval for the relative risk was calculated according to a method developed for clinical trials [Simon, 1986] (III).

3.2 Estimates on sampling variation at low template concentrations

For statistical estimation of the absolute amounts of template molecules present in an aliquot pipetted from a sample of a known concentration (IV, V), a normal distribution was assumed for templates present at a level of 40 copies or more whereas the Poisson distribution was applied for calculations concerning less than 40 template copies. The probability of a certain ratio of wild type to reference templates to be present in an aliquot containing a known ratio of templates was calculated by multiplying the distribution-based estimated probabilities for each possible absolute amount of wild type and reference templates present in the sampled aliquots. As the same ratio can arise from different combinations of absolute copynumbers of the respective templates, the probability of a certain ratio was estimated by summing the probabilities of the different copynumber combinations resulting in the same ratios. The probability distributions and confidence intervals of a ratio of two templates in an aliquot was estimated from the cumulative probabilities of the most frequent ratios.

Results and Discussion

1. Design of the RT-PCR assay for accurate quantification of relative transcript levels

1.1 Principles for detection and quantification of SCCA mRNA levels

We have utilized the tissue-specific expression of the SCCA1 and SCCA2 genes to detect epithelial cells in blood and to measure the expression levels of the two genes in tissue samples containing a mixture of cell types. Non-quantitative RT-PCR amplification was used to detect occult epithelial cells in the circulation of patients with cervical cancer (I) according to the principle described by Smith and colleagues [Smith, 1991], and a quantitative RT-PCR was developed in order to study the relative expression levels of the SCCA genes in tumor specimens of head and neck cancer patients (II, III). The latter method has been used with slight modifications to study factors affecting the quantitative accuracy of sensitive amplification assays at different levels of input template (IV, V). The principle of quantifying the expression levels of highly homologous transcripts in relation to each other was originally described for fibrillin alleles in patients with Marfan's syndrome [Karttunen, 1996]. As highly homologous mRNA transcripts are likely to be similarly affected by nonspecific factors involved in mRNA degradation, the relative level of the transcripts is expected to remain fairly constant despite sample to sample variations in RNA degradation during handling and storage. In addition the two transcripts are likely to be subjected to similar variations in efficacy of RNA extraction procedures and during reverse transcription. The 98% homologous [Schneider, 1995] and tissue specifically expressed [Cataltepe, 2000] SCCA genes have provided suitable targets for such a quantitative setup.

The equivalence of replication efficiencies has been shown by mathematical modeling to be the key feature for achieving optimal quantitative precision in co-amplification based PCR systems [Nedelman, 1992]. In accordance with this, amplification of internal standards and wild type templates with common primers has been unequivocally shown to yield superior quantification accuracy than amplification of standards and wild type templates with separate primers [Raeymaekers, 2000]. We have striven to take the concept of equivalence of replication efficiencies further yet by designing PCR primers common for SCCA1, SCCA2 and the internal standards that produce amplicons of equal length and close to identical base pair composition. This way secondary structures caused by folding of the single stranded PCR amplicons after denaturation are unified, and subsequently the differences in annealing and elongation efficiencies of the primers to the different target templates are minimized. To optimize PCR primer hybridization features and PCR product nucleotide composition and length, 22 primers equally specific to SCCA1 and SCCA2 were designed, synthesized and tested under different cycling conditions. With the primers chosen for the final assay for SCCA mRNA (II, III, V) the amplicons of SCCA1, SCCA2 and an internal standard were coamplified, producing a single 108 bp

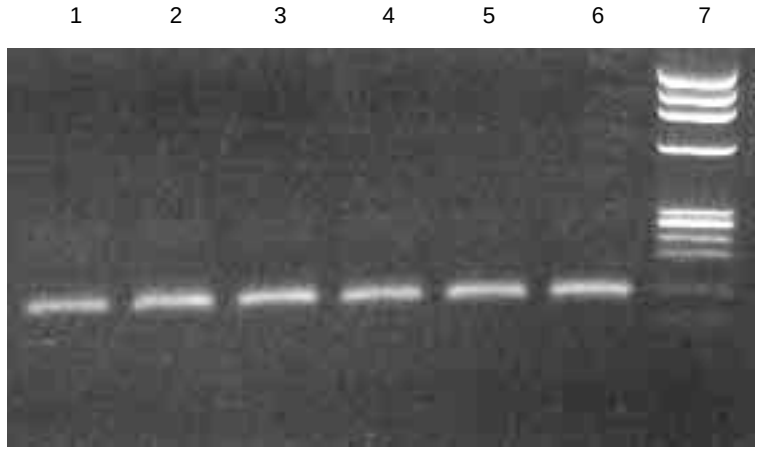

Figure 12. Coamplification of SCCA1, SCCA2 and internal standard templates. Ethidium bromide stained agarose gel displaying the single 108 bp band including all three amplicons. All nested PCR reactions were amplified to the plateau. In this way the absolute amount of PCR product in the reactions originally containing various amounts of target template, was unified to fit into the dynamic range of the minisequencing detection assay. The close-to-identical reaction kinetics of the three templates enabled accurate quantification of their relative levels in the endpoint PCR products. Lanes 1–6: patient tissue samples consisting of five 10 μm microscopic frozen sections. Lane 7: phiX-DNA marker.

amplicon. These primers amplified the target templates over a ±5 °C range of annealing temperatures. With the final reaction conditions no primer dimers or other non-specific amplification could be visualized by ethidium bromide staining (fig. 12).

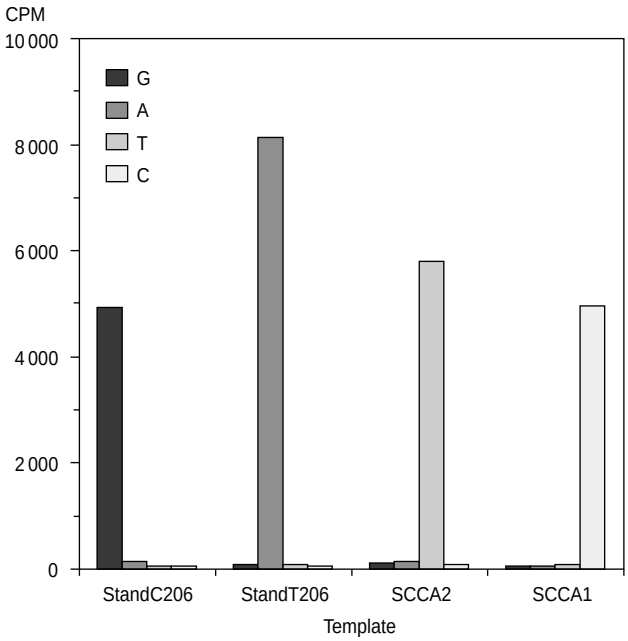

Figure 13. Capacity of the minisequencing assay to discriminate between templates differing at single nucleotide position. The PCR products derived from the SCCA1, SCCA2 and two internal standards (StandC206 and StandT206) differing at position 206 of the SCCA2 cDNA sequence were mixed and minisequenced with with four different ^{3}H-labelled nucleotides (dGTP, dATP, dTTP and dCTP).

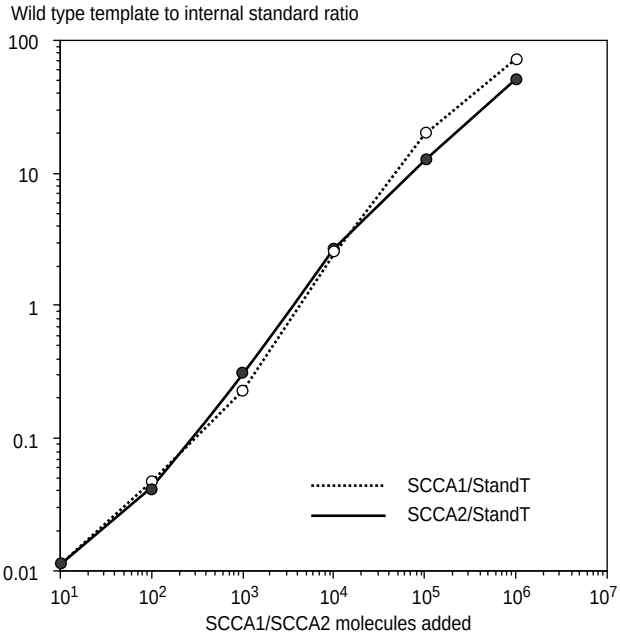

Figure 14. Dynamic range of the RT-PCR assay for quantifying the relative levels of SCCA and internal standard. When mixing 10 000 molecules of the internal standard (Stand T) with 10-fold dilutions of the SCCA1 and SCCA2 transcripts the dynamic range was 6 orders of magnitude.

1.2 Measuring range

In order to determine the measuring range of assay, we amplified dilutions of SCCA1 and SCCA2 cDNA in a 1:1 ratio over a concentration range of 10^2 to 10^9 copies together with different amounts of internal standard (publication II, fig. 1). The ratio of SCCA1 and SCCA2 PCR products remained constant when the amount of wild type templates exceeded that of the internal standard. This resulted in a dynamic range of six orders of magnitude when using more than 1000 molecules of internal standard in the reactions (fig. 13). When the assay was optimized for extreme sensitivity, using 20 molecules of internal standard and in vitro transcribed cRNA templates, the measuring range was limited to 4 orders of magnitude (publication V, fig I). We strived to gain a wide measuring range by systematically amplifying all nested PCR reactions to the plateau phase. By doing this, the endpoint PCR product is brought into the dynamic range of the minisequencing endpoint detection assay, irrespectively of the absolute amount of input wild type templates. Quantitative detection on the or plateau phase of amplification is possible in coamplification systems provided that the amplification efficiencies of the coamplified templates are equal [Raeymaekers, 2000]. In such a setting the amplification efficiencies of the different templates will be equally affected by factors limiting exponential amplification and the ratio of the templates will remain unchanged despite saturation of the reaction. The minisequencing assay provided accurate discrimination of single nucleotide sequence differences in the coamplified SCCA1, SCCA2 and internal standard PCR products (fig. 14). In addition, the assay has an exceptional signal to noise ratio which is explained by the great difference in binding affinity between labeled nucleotides enzymatically incorporated to an oligonucleotide primer and non-specific adsorption of nucleotides to any of the components present on the solid phase.

1.3 Quantitative accuracy

The accuracy of the assay for measuring relative levels of SCCA1 and SCCA2 mRNA (II, III) was determined from test samples of pooled cDNA from six normal epithelium

samples and six squamous cell carcinoma tumor samples from the head and neck region. The test samples were included in ten individual sample series and analysed in separate experiments as single samples. The 99% confidence intervals of the means were 0.23 to 0.27 (mean=0.25) and 0.37 to 0.41 (mean 0.39) for the normal epithelium and squamous cell carcinoma tumor test samples, respectively (publication II, fig. 2). The inter-assay coefficient of variation (CV) was 9% for the normal, and 5% for the tumor test samples. 1000 copies of a non-linearized plasmid containing the internal standard sequence was included with the wild type SCCA 1 and SCCA2 templates in the amplification reactions resulting in co-amplification of the three templates. The main function of the internal standard was to ensure that sufficient amounts of the wild type templates were present to yield an accurate ratio of the two wild type templates (II, III, V). The internal standard also served as a quality control, as a decrease in amplification of the standard not accompanied by an increase in amplification of the wild type templates would indicate the presence of inhibition or nonspecific competition in the PCR reactions. When optimizing the assay for extreme sensitivity (V) we observed that the degree of cross-contamination of negative samples was efficiently avoided when as little as 20 molecules of internal standard was used (V). This could indicate that amplification from contaminating DNA or RNA often is derived from single or minimal amounts of template molecules that can be competitively suppressed by even as little as 40 single stranded copies of an internal standard.

The concept of using a highly homologous reference template enables highly accurate and reproducible quantification over a wide range of input template concentrations. This technique is not restricted to the endpoint detection by minisequencing, but can be used in any quantitative system capable of distinguishing highly homologous PCR products. Kinetic or real-time PCR technology has become the mainstream methodology for quantitative applications mainly due to simplified assay procedures and diminished risk of cross-contamination of samples. Combining a detection technology capable of distinguishing single nucleotide differences such as molecular beacons [Tyagi, 1996] to kinetic PCR, NASBA or other alternative kinetic amplification techniques would produce a versatile technology for accurate quantification of rare mRNA transcripts. Thus the benefits of competitive and kinetic amplification approaches could be combined.

1.4 Stochastic considerations

When optimizing the RT-PCR assay for maximal sensitivity (V) we noted that the variation in quantitative accuracy increased dramatically when less than 1000 copies of target template molecules were present. This phenomenon has earlier been termed the Monte Carlo effect [Karrer, 1995]. The observed variation was however much greater than suggested by estimations based on normal and Poisson distributions that have been used in limited dilution assays [Sykes, 1992]. In order to evaluate to what degree the observed variation depended on methodological inaccuracy and to what degree it could be attributed to stochastic sampling variation, we developed an algorithm for calculating the stochastic variation of the resulting ratio of two templates when sampling from a solution containing the two templates at a known ratio (IV). This algorithm, taking in account the multiplicative effect imposed by the variation in the absolute numbers of both wild type and reference template copies, predicted close to 10-fold differences when aiming at sampling an aliquot containing 8 copies of each of the templates. This variation decreases rapidly when the amount of templates increases, being largely negligible at levels above 1000 molecules per reaction (fig. 15, publication III: fig. 1 and table 1). In order to compare the statistically estimated sampling variation with the empirically observed variation, we co-amplified a 5-fold dilution series of mRNA from SCCA-producing A431 cells corresponding to approximately 8–1000

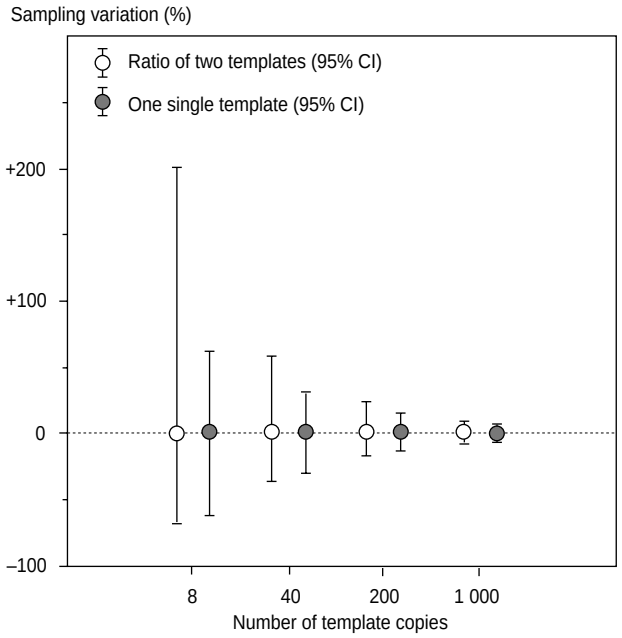

Figure 15. Variation imposed by stochastic distribution of rare template molecules. The 95% confidence intervals of the sampling variation is shown separately for low concentrations of single templates and for the ratio of a target and reference template.

SCCA mRNA transcripts, with 20 molecules of a double stranded DNA internal standard. This experiment was repeated 12 times and the 95% confidence intervals were calculated for each sensitivity level and compared with the corresponding confidence intervals of the statistically predicted sampling variation. We observed that stochastic factors affecting sampling explain a major part of the great variation observed when quantifying relative levels of two rare templates (publication V: fig III).

2. Clinical Observations

2.1. Detection of SCCA mRNA in peripheral blood

Circulating epithelial cells were detected in six of 15 cervical cancer patients under follow-up using a non-quantitative RT-PCR for SCCA (I). Of the patients testing positive, five had a diagnosis of epidermoid carcinoma and one had an adenomatous carcinoma (publication I, table 1). Four patients experienced recurrent disease during a 24-month follow up period and three of these had tested positive in the RT-PCR for SCCA while still in remission. One of the patients did not, however, test positive despite progressive disease at the time of sampling. Interestingly, two of 24 control subjects tested positive, both being pregnant women at term (publication I, table 2). This finding is possibly explained by release of squamous epithelial cells due to proteolytic processes in the uterine cervix during the last days of pregnancy. Due to the small number of patients in this study, no conclusions regarding the prognostic potential of this technique could be made. This study however, clearly showed that the tis-

sue-specific expression of squamous cell carcinoma antigen can be used to detect occult epithelial cells.

The sensitivity of the assay was determined to be 10 cells among 10^6 leukocytes as defined by cell spiking experiments with cultured A431 epidermoid carcinoma cells. A total of 4 PCR reactions were amplified from each of the blood samples using a nested primer protocol. The result was considered positive if the correct PCR product was amplified in one or more of the PCR reactions. The non-quantitative assay setup did not allow definitive exclusion of the possibility that some positive signals would have been derived from low-level background expression in leukocytes. The minimal amount of specific template present in the samples is reflected by the fact that we were not able to amplify the SCCA derived amplicon in more than two of four reactions from any of the patient samples. It is possible that shedding of malignant cells in circulation is not a continuous phenomenon, but rather occurs at intervals. In addition the circulation time of cells of epithelial origin before being trapped in the capillary beds of the lung, liver or other peripheral sites might be relatively short as the total blood volume is circulated on an average once each minute in a person at rest [Despopoulos, 1986].

The observation that cells were released from the cervix during the last days of pregnancy prompted us to develop a competitive RT-PCR for quantitative detection of minimal levels of the SCCA1 and SCCA2 transcripts in blood samples obtained in connection with invasive therapy of squamous cell carcinomas (V). The original aim was to evaluate the effect of invasive therapy on cell-shedding into circulation. We chose to study head and neck cancer patients undergoing radical surgery and cervical cancer patients undergoing intracavitary radiation therapy to the uterine cervix. Especially the latter setting was anticipated to bring about at least temporary shedding of epithelial cells into circulation as the intervention is relatively traumatic and includes no restriction of the blood flow to or from the cervical region. The technique allowed reproducible detection of 40 copies of SCCA cRNA template spiked in total RNA from 1μg of total leukocyte RNA (publication V: figure 1). We did not however observe a rise in the level of SCCA mRNA following invasive treatment in any of the head and neck or cervical cancer patient samples (unpublished results).

The concept of detecting occult malignant cells in peripheral blood by RT-PCR based amplification of tissue-specific gene transcripts was first described in 1991 [Smith, 1991]. During the passed decade a great number of techniques have been described using tyrosinase [Smith, 1991], cytokeratins [Ko, 2000], PSA [Jaakkola, 1995; Henke, 1997], PSM [Lintula, 1997] and many other genes as targets for tissue specific RT-PCR amplification. Early attempts were based on non-quantitative techniques and highly variable results were often referred to as illegitimate expression by leukocytes and the existence of expressed pseudogenes. Despite the expectations that real-time PCR technology would solve these problems, as an increase in expression of the target gene could be quantitatively distinguished from baseline expression, whatever the source, results are still quite modest. To date, none of these techniques have reached clinical practice. We conclude that the major source of the variability observed with this type of techniques are in fact due to the low and variable amounts of occult malignant cells present in the peripheral circulation at any given time point.

2.2. Relative levels of SCCA2 and SCCA1 mRNA in head and neck tumor biopsies

Due to the low level of target template in the blood samples we turned the focus to developing accurate techniques to quantify gene expression in microscopic tissue sections. The finding that the neutral and acidic isoforms of SCCA in fact were products of two separate genes, SCCA1 and SCCA2 [Schneider, 1995] spurred us to develop a method that would enable accurate quantitation of the rela-

tive expression levels of these two highly homologous genes. This led to the development of an RT-PCR based technique that enabled accurate quantification of the relative levels of SCCA1 and SCCA2 mRNAs occurring in biopsy specimens of tumor tissue and normal epithelium. The measuring range of the assay (II, III) was 10^3–10^9 copies of input wild type cDNA template (publication II: figure 1). In our material of 117 samples from microscopic frozen sections of normal epithelium and tumor specimen 16 samples were excluded, as the observed SCCA2/SCCA1 ratio was derived from less than one thousand copies of cDNA template. All observed CPM signals for SCCA1 and SCCA2 in the accepted samples exceeded three times the baseline determined from their respective negative controls.

We applied this method to study a material of banked tumor tissue specimens including 30 primary head and neck tumors and 11 recurrent tumors from patients of which follow up data was available from clinical records (publication III: tables 1 and 2). We observed that the relative levels of SCCA2 to SCCA1 mRNA was higher in all tumor groups than in normal epithelial samples. In addition the relative levels of SCCA2 to SCCA1 mRNA was significantly higher in recurrent tumors as well as in primary tumors that had eventually recurred in course of time, than in primary tumors of patients not experiencing a recurrence (publication III: fig. 1). Statistical analysis of recurrence free survival revealed a relative risk of 7.2 (CI 1.2-13.3) of developing recurrent disease in patients with a primary tumor expressing mRNA of SCCA2 at a high level in relation to SCCA1 (publication III: fig. 2). When analyzing other commonly used prognostic indicators in head and neck cancer, the presence of metastases in regional lymph nodes was in addition to a high SCCA2 to SCCA1 mRNA ratio in the primary tumor, the only independent predictor of disease recurrence in our material. There was however no correlation between an elevated SCCA2 to SCCA1 mRNA ratio and the presence of lymph node metastasis, indicating that these two prognostic variables reflect different malignant features of the tumors.

Inoculation of tumors with an antisense SCCA mRNA construct suppressing the expression of both SCCA1 and SCCA2 has been shown to suppress tumor growth in vivo. [Suminami, 2001] This effect has been suggested to be partly explained by the ability of SCCA1 to inhibit migration of NK-cells. Tumor cells transduced with SCCA1 cDNA have also been shown to be more resistant to induced apoptosis as compared to control cells in vitro, and also to form larger tumors than corresponding controls in nude mice. [Suminami, 2000] We observed a relative increase in SCCA2 expression in relation to SCCA1 in tumors with a poor outcome. The method used for relative quantification does not, however, exclude the possibility that the absolute levels of both SCCA1 and SCCA2 were elevated in these cases. As the number of clinical samples for which follow up data was available was limited, the power of our statistical analyses remains fairly weak. Our results, however, indicate that in addition to an absolute rise in the expression of the SCCA genes, also a relative increase in the expression of SCCA2 could play a functional role in the pathophysiology of squamous cell carcinomas.

Summary and Conclusions

We have been able to show that highly accurate mRNA quantification can be achieved by comparing the levels of closely related transcripts to each other. The concept of using a closely related mRNA transcript as a reference template for quantification has been demonstrated in a competitive RT-PCR based technique. This technique as such is unlikely to be directly applied as a tool for mRNA based grading in the future due to the relatively complicated methodology. The concept of relative quantification of closely related mRNA:s is however readily applicable to a real-time PCR setup as well as to other amplification- and detection technologies.

We were able to demonstrate the effect of stochastic sampling variation in amplification systems at different template concentrations. These effects are equally present regardless of quantification technology, but are greatly are pronounced at low levels of input template when quantification is done against a reference template. Below a level of 1000 template molecules the stochastic distribution of target molecules during sampling is a major source of variation. As the sensitivity of RT-PCR is sufficient for quantification of mRNA expression from even single cells, accurate quantification is possible only for highly expressed targets. For most gene transcripts, RT-PCR remains a qualitative technique at the single cell level.

The relative levels of SCCA1 and SCCA2 mRNA in squamous cell tumors of the head and neck region was demonstrated to correlate with tumor types displaying an aggressive behavior as well as with the recurrence free survival of the patients. These consistent but subtle variations in the relative levels of the SCCA genes were beyond the quantitative accuracy of the most commonly used techniques for mRNA quantification. As the number of patient samples with available follow up data was limited in our study, the clinical applicability of the SCCA2/SCCA1 mRNA ratio as a molecular marker in head and neck cancer remains to be verified. For the time being, measurement of this parameter remains a potential target for future grading techniques for squamous cell carcinomas of the head and neck region and possibly of other sites.

Using a sensitive non-quantitative RT-PCR assay for SCCA mRNA we were able to detect occult epithelial cells in the circulation of cervical cancer patients. In an attempt to use a quantitative method capable of quantifying down to 40 copies of mRNA template, that would distinguish expression from occult epithelial cells from a possible baseline expression in blood cells, we could not however reliably detect malignant cells in the samples. It is likely that detection of mRNA from malignant cells in the peripheral circulation is hampered by stochastic considerations due to low and variable levels of occult cells in circulation at a given time point. This phenomenon equally concerns detection techniques based on enrichment of malignant cells with immunological or gradient centrifugation techniques. RT-PCR provides a potential technology to detect and quantify occult cells at sites where they are enriched over a period of time, such as lymph nodes, bone marrow aspirates and possibly also leukapheresis harvests. The use of RT-PCR for detecting occult cells in peripheral blood cannot, however, be considered feasible in a clinical setting.

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