A Predictive Model of Rectal Tumour Response to Pre-operative High-Dose Rate Endorectal Brachytherapy

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List of Abbreviations

APAF-1	Apoptosis Protease Activating Factor-1
ATP	Adenosine Triphosphate
CARD	Caspase Recruitment Domain
CART	Classification and Regression Tree
CR	Complete response
CRC	Colorectal Carcinoma
CT	Computerized Tomography
DNA	Deoxyribonucleic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EUS	Endorectal Ultrasonography
H&E	Haematoxylin and Eosin
HDREB	High Dose Rate Endorectal Brachytherapy
HIF-1α	Hypoxia- Inducible Factor-1 alpha
ICC	Intra-Class Correlation Coefficient
IHC	Immunohistochemistry
IL-1	Interleukin-1
IL-6	Interleukin-6
kDa	Kilodalton
MAP	Mitogen Activating Protein
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
NR	No response
PDGF	Platelet Derived Growth Factor
PI3-kinase	Phosphotidolinositol-3 kinase
PR	Partial response
RHAMM	Receptor for Hyaluronic Acid Mediated Motility
ROC	Receiver Operating Characteristic
TGF-α	Transforming Growth Factor-alpha
TGF-β	Transforming Growth Factor-beta
TMA	Tissue Microarray
TUNEL	Terminal Deoxynucleotidyl Transferase End Labeling
VEGF	Vascular Endothelial Growth Factor
VEGFR2	Vascular Endothelial Growth Factor Receptor 2

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Abstract

Pre-operative radiotherapy for patients with locally advanced rectal carcinoma has been shown to improve survival rates and local tumour control. The ability to identify tumours most likely to undergo a complete or partial response would improve the selection of patients for radiotherapy and potentially modify post-treatment planning. The aim of this study was to develop a multi-marker model of tumour response to pre-operative high-dose rate endorectal brachytherapy (HDREB). Immunohistochemistry (IHC) for p53, Bcl-2, VEGF, APAF-1 and EGFR was carried out on 104 pre-treatment rectal tumour biopsies from patients undergoing a pre-operative HDREB protocol. Immunoreactivity was scored by at least three pathologists using a semi-quantitative scoring method. The reproducibility of the scoring system was evaluated. Receiver operating characteristic curve (ROC) analysis was performed for each protein to determine clinically relevant cut-off scores for defining tumour positivity. Multivariate logistic regression analysis was carried out to identify independent predictive factors of tumour response. Both the semi-quantitative scoring system and ROC curve analysis were found to be reproducible. In addition, the combined analysis of VEGF and EGFR was highly predictive of complete pathologic response to radiotherapy. EGFR was found to independently predict complete or partial tumour regression but only with low sensitivity and specificity. A large-scale prospective study is necessary to confirm these findings. Moreover, the novel methodology proposed and validated in this study to assess immunoreactivity could significantly enhance the value of IHC findings in colorectal cancer as well as other tumour types.

Résumé

La radiothérapie pré-operatoire contre le cancer localisé et avancé du rectum prolonge la survie et améliore le contrôle local de la tumeur. L'identification des tumeurs dont la probabilité de regression complète ou partielle est elevée faciliterait la sélection de meilleurs candidates et pourrait avoir un impact sur la planification de traitment post-thérapie. Le but de cette étude était de développer un modèle multi-marqueurs de regression suite à la brachythérapie pre-opératoire endorectale à haute-dose (HDREB). L'immunohistochimie (IHC) pour les protéines p53, Bcl-2, VEGF, APAF-1 and EGFR fut effectuée sur 104 biopsies de tumeur rectale prises avant le traitment HDREB. L'expression de chaque protéine fut evaluée par au moins trois pathologistes utilisant un système d'évaluation semi-quantitatif. Le degrée de reproduction de ce système fut déterminé. L'analyze des courbes Receiver Operating Characteristic (ROC) fut utilisée afin de déterminer le score le plus approprié au delà duquel l'expression de la protéine est considerée positive. La regression logistique multivariée fut employée afin d'identifier les facteurs prédictifs independents. Le système d'évaluation ainsi que l'analyze ROC furent reproductibles. De même, la combinaison de VEGF et EGFR fut la plus importante pour prédire la regression pathologique complète de la tumeur. EGFR fut l'unique facteur prédictif de regression complète ou partielle mais la sensibilité et la spécificité étaient peu élevées. VEGF et EGFR ensemble ont une valeur prédictive en tant que marqueurs de regression complete suite à la HDREB. Une étude prospective à grande échelle serait nécessaire afin de confirmer ces résultats. De plus, la nouvelle méthode proposée et validée au cours de cette étude pour évaluer l'expression des protéines détectée par l'IHC pourrait améliorer l'utilité clinique des résultats obtenus utilisant cette technique.

Contribution of Authors

Most experimental work and statistical analyses for this project were carried out by me under the supervision of the senior authors and external advisor Dr. Russell Steele. CART analysis was performed by Dr. Steele whereas immunohistochemistry for RHAMM and EGFR was carried out at the Institute of Pathology, University Hospital of Basel, Switzerland and the Jewish General Hospital of Montreal respectively. I participated significantly in the conception, design and writing of each paper included in this thesis. The final submission of each manuscript was overseen by the senior authors.

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CHAPTER 1: Introduction and Literature Review

1.1 Introduction

Colorectal carcinoma is a leading cause of cancer-related mortality in North America and Western Europe [1]. In 2002 more than 1 million people developed this disease worldwide accounting for about one tenth of all cancers [2]. Although early stages of the disease are linked to excellent post-operative prognosis and a cure rate of 80%-95%, approximately 90% of patients with newly diagnosed cancers present with locally advanced tumours and lymph node metastasis reducing the 5-year survival rate to 25%-60% [3].

Several randomized trials have described a significantly improved clinical outcome in patients treated with pre-operative radiotherapy compared to surgery alone [4-6]. The Swedish Rectal Cancer Trial reported a significant decrease in local recurrence rates and improved survival in patients receiving pre-operative short-term (5 x 5 Gy fractions) radiotherapy [7, 8]. The Stockholm I and II trials assessed short-course pre-operative radiotherapy versus surgery alone and found a significant reduction in local recurrence rates in the group given neo-adjuvant radiotherapy [9, 10]. Along with total mesorectal excision, pre-operative radiotherapy was also found to improve local control and prognosis [11, 12].

Pre-operative high-dose rate endorectal brachytherapy (HDREB) is a novel form of radiotherapy administered to patients with locally advanced resectable rectal cancer [13]. This treatment differs significantly from standard radiation protocols as a high dose fraction (6.5 Gy) is given once daily over 4 days. Surgery is performed four to eight weeks after irradiation. Pre-operative HDREB, though still an experimental approach, has

demonstrated high rates of tumor downstaging and complete pathologic response [13]. Tumour regression grade following irradiation is linked to improved disease-free survival and decreased local failure [14, 15].

Currently there are no clinically reliable predictors of colorectal tumour response to pre-operative radiotherapy [16]. However, several cellular processes have been identified as important promoters or mediators of radio-sensitivity and tumour response following pre-operative radiotherapy including apoptosis, tumour cell proliferation and angiogenesis [17-19].

1.2 Apoptosis

Apoptosis, or programmed cell death, is necessary for physiological and developmental processes in normal human tissue [20]. Imbalance of apoptosis seems to contribute significantly to the pathogenesis of colorectal cancer [19, 21, 22]. Several studies have reported that the proportion of epithelial cells undergoing apoptotic cell death, frequently called the apoptotic index, increases with tumour progression from adenoma to carcinoma. Apoptotic indices of 1.5% to 2.46% have been observed in carcinomas while significantly lower values (<1.0%) are found in earlier lesions and normal mucosa [23-25].

Ionizing radiation induces apoptosis primarily through the mitochondria-mediated pathway [26]. The ratio of pro- and anti-apoptotic proteins such as apoptosis protease activating factor-1 (APAF-1) and Bcl-2 determines the relative permeability of the mitochondria to cytochrome c, which can initiate a cascade of apoptotic events ultimately

resulting in cell death [19, 26]. The tumour suppressor gene p53 can regulate apoptosis by mediating the expression of both APAF-1 and Bcl-2, and has been extensively studied in colorectal cancer [19, 26].

1.2.1 APAF-1

APAF-1 is a 130 kilodalton (kDa) protein that plays a central role in the activation of caspases involved in mitochondria-mediated apoptosis [27]. The APAF-1 protein consists of 3 domains: the N-terminal caspase recruitment domain (CARD), the CED-4-like domain responsible for nucleotide binding and the C-terminal domain containing multiple repeats or tryptophan and aspartate residues (WD repeats) essential for carrying out protein-protein interactions [27]. Cytochrome c released from the mitochondria following apoptotic stimuli binds to the WD region of the APAF-1 protein [28]. In the presence of ATP, conformational changes of the WD region unmask the CARD domain allowing the binding of pro-caspase-9. Oligomerization of the APAF-1 protein ensues through its CED-4 like domains creating a 7-spoke wheel-like structure called the apoptosome [28]. Subsequent activation of pro-caspase-9 by autocatalytic cleavage initiates a cascade of downstream effector caspases leading to apoptosis [28].

APAF-1 plays an important role in developmental programmed cell death [29]. *APAF-1 -/-* mice suffer from birth defects as well as from severe craniofacial abnormalities, retention of interdigital webs, brain over-growth due to hyper-proliferation of neuronal cells, and abnormal development of the eye and inner ear development [29]. Absence of APAF-1 protein appears to prevent activation of caspase-3 *in vivo* and to impair processing of

caspases -2 and -8 leading to cellular resistance to apoptotic stimuli such as radiation [30, 31].

APAF-1 appears to act as a tumour-suppressor gene [27]. Mustika *et al.* described intense and diffuse cytoplasmic immunohistochemistry (IHC) staining for APAF-1 in normal skin, nevi and melanoma *in situ* [32]. Weaker, focal positivity was observed in melanoma and in less than 25% of all tumour cells from metastatic melanoma suggesting a role for APAF-1 in disease progression. Additionally, an inverse correlation between APAF-1 expression and pathologic stage has been reported in this disease [27]. Loss of heterozygosity at the *APAF-1* locus (12q22-23) has been correlated with decreased mRNA expression in metastatic melanoma as well as with poor disease outcome and chemo-resistance [33]. In colorectal cancer an increased frequency of allelic imbalance at the *APAF-1* locus has been associated with tumour progression from adenoma to carcinoma to metastatic cancer [34].

A study by Robles *et al.* demonstrated that APAF-1 may be an essential component of p53-mediated apoptosis [35]. p53 mutation and APAF-1 expression were found to be inversely correlated in melanoma cell lines [36]. The predictive value of APAF-1 to pre-operative radiotherapy has not yet been investigated.

1.2.2 Bcl-2

The Bcl-2 family includes both pro- and anti-apoptotic members including Bax and Bcl-2 [37]. Bcl-2 is an intracellular integral protein localized to the nuclear envelope, the outer

mitochondrial membrane and the endoplasmic reticulum. High levels of Bcl-2 protein were first detected by IHC in follicular and B-cell lymphomas with the translocation t(14;18); the gene was isolated thereafter [38]. Bcl-2 is an anti-apoptotic protein whose function is to maintain the integrity of the mitochondrial outer membrane and inhibit the release of cytochrome c thereby preventing apoptosis [37]. The over-expression of Bcl-2 is widely observed in human cancer cells; however its association with prognosis appears to be tumour specific [38]. Poor prognosis is generally observed in Bcl-2-expressing tumours from patients with acute myeloid leukemia, diffuse large B-cell lymphomas, prostate and ovarian cancer [39]. However in other neoplasms such as lung, thyroid and breast carcinomas, Bcl-2 over-expression confers a favourable patient outcome [39].

Over-expression of Bcl-2 is frequently found in colorectal adenomas and is significantly decreased with malignant transformation [40]. In addition, an important reduction in Bcl-2 expression with more advanced Dukes' stage has been reported [40, 41]. Numerous studies have described an association between increased Bcl-2 immunoreactivity and improved survival time [41-43] whereas other groups have found no link with prognosis [44, 45]. Several reports have identified an interaction between Bcl-2 over-expression and p53 staining with Bcl-2 positive/p53 negative tumours demonstrating superior outcomes compared with Bcl-2 negative/p53 positive tumours [22, 42, 46, 47]. This combined analysis has also shown to correlate with local recurrence, invasion and metastasis [41, 48].

In prostate cancer, a high Bcl-2/Bax ratio was correlated with an increased risk of failure following radiotherapy [49]. Over-expression of Bcl-2 in cervical cancer, bladder cancer

and squamous cell carcinoma of the larynx was significantly related to radio-resistance [50-52]. Though the majority of studies on Bcl-2 in colorectal cancer prior to pre-operative radiotherapy or chemo-radiotherapy have not shown a correlation with expression and tumour response [53, 54], the predictive value of Bcl-2 has been reported by other groups particularly in combined analysis with Bax or p53 [55].

1.2.3 p53

The *p53* gene plays a pivotal role in the cellular response to DNA damage [56]. p53 can inhibit cellular proliferation and regulate both cell-cycle arrest and apoptosis through the transcriptional activation of downstream effector genes such as *p21*, and pro-apoptotic genes *PUMA*, *Noxa* and *Bax*. Furthermore, p53 has been shown to directly induce permeabilization of the outer mitochondrial membrane by forming a complex with the anti-apoptotic protein Bcl-2, resulting in cytochrome c release which leads to apoptosis [57]. As described previously, p53 also appears to regulate cell death by directly activating *APAF-1* [35]. A role for p53 has also been demonstrated in DNA repair [58]. p53 binds directly to sites of damage and can up-regulate *GADD45* [59]. Wild-type *p53* has additionally been implicated in angiogenesis through activation of genes regulating new blood vessel formation such as Vascular Endothelial Growth Factor (VEGF) [60].

Loss of wild-type p53 protein is reported in approximately 70% of colorectal cancers and occurs early in the adenoma-carcinoma sequence in sporadic tumours [19]. p53 mutations are correlated with tumour aggressiveness, poor local control, advanced disease stage, lymph node metastasis, and increased risk of distant metastasis in the majority of studies

[38, 41, 48, 61-65]. Mutation of *p53* detected by DNA sequencing is associated with poor survival in rectal cancer [66, 67]. However results based on immunohistochemical analysis of p53 protein and prognosis are conflicting [41, 44, 68-71]. The immunohistochemical detection of mutant p53 is based on the premise that the half-life of the wild-type p53 protein is short. Mutant p53, with its significantly longer half-life, accumulates in the cell and can therefore be visualized [58]. Several studies have described severe discordance between immunohistochemical findings and mutational analysis by DNA sequencing [63, 72]. In addition, the numerous scoring methods used to describe p53 "positivity" in colorectal tissue following IHC may contribute to these conflicting reports [73].

Radio-responsiveness and p53 status has been extensively studied. Although the over-whelming majority of reports show that presence of wild-type p53 is associated with sensitivity to irradiation, radio-response appears to be tissue specific [58, 63]. Loss of p53 function has been shown to impact in three ways: first, on radio-sensitivity by decreasing apoptosis and mitotic cell death, second, on the repopulation of tumour cells following radiotherapy by promoting increased cell proliferation and decreased growth factor dependency and third, on tumour re-oxygenation by increasing survival under hypoxic conditions and altering angiogenesis [58].

In rectal cancer, mutation of *p53* detected by DNA sequencing is associated with decreased tumour response to pre-operative radiotherapy [67, 72]. p53 appears to have an impact on tumour shrinkage and histologic regression after irradiation [74]. However, no

consensus has been reached on the predictive value of p53 expression assessed by means of IHC [53, 54, 75-80].

1.3 Epidermal Growth Factor Receptor (EGFR)

EGFR is a 170 kDa transmembrane protein whose primary ligands, Epidermal Growth Factor (EGF) and Transforming Growth Factor-α (TGF-α) are known activators of DNA synthesis and cell growth [81]. Ligand binding produces dimerization of the receptor, auto-phosphorylation and activation of intrinsic protein tyrosine kinase activity leading to the transduction of signaling pathways involved in proliferation, cell division and differentiation [81]. The mitogen activating protein (MAP) kinase and AKT signaling pathways have been found to mediate intracellular EGFR signaling [81]. The biologic responses to MAP kinase induction result in increased expression of proteins governing cell-cycle regulation. AKT, an anti-apoptotic kinase, is implicated in cell survival and promotion of angiogenesis and has also been linked to activation of matrix metalloproteinases facilitating tumour growth and promotion [82, 83].

In colorectal cancer, EGFR over-expression detected via IHC is observed in approximately 50-70% of tumours [84-89] and has been linked to tumour progression, including advanced tumour stage, an increased risk of liver metastasis, extramural vascular and perineural invasion and possibly worse survival [87, 90-92]. Although the role of EGFR as a prognostic factor remains unclear [86, 93-96] its ability to promote aggressive tumour behavior has made EGFR an interesting target for therapeutic intervention [97, 98].

Ionizing radiation is known to initiate activation of EGFR and its downstream signal transduction pathways within minutes of exposure [99]. The activation of Ras has been shown to directly contribute to increased intrinsic resistance in human tumour cell lines following exposure to low doses of radiation [100]. MAP kinase activation mediates cell proliferation after single and repeated exposures to low doses (1.6-1.8 Gy) of ionizing radiation [100, 101] and appears to represent the likely molecular mechanism underlying accelerated repopulation [99]. The selective inhibition of Ras, PI3-kinase and AKT increases sensitivity to radiation in human colon cancer cell lines [97, 101]. Inhibition of EGFR has been linked to radio-sensitization [99].

Few studies have investigated the IHC expression of EGFR *in vivo* and its value as a predictive marker of tumour response to pre-operative radiotherapy. Giralt *et al.* studied EGFR IHC expression in 45 pre-treatment rectal tumour biopsy specimens, and found a negative effect of EGFR expression on tumour response [102]. In a larger study of 85 patients by the same group, positive EGFR expression was associated with lack of complete tumour regression [103]. A high level of EGFR expression was linked to decreased tumour downstaging after pre-operative chemo-radiotherapy in a study on 183 patients [104].

1.4 Angiogenesis

Angiogenesis is the formation of new blood vessels from pre-existing vascular networks [105]. VEGF is considered one of the most potent mediators of angiogenesis involved in both normal physiology and pathology [106]. Evidence suggests that VEGF is vital for

embryonic development and survival in post-natal life [107]. Loss of both VEGF alleles results in near complete absence of vasculature in embryos [107]. Inhibition of VEGF by gene-targeting has been shown to result in increased mortality, stunted body growth and impaired organ development [107]. VEGF is involved in endochondral bone formation, a fundamental mechanism for longitudinal bone growth. VEGF mRNA is expressed by hypertrophic chondrocytes in the epiphyseal growth plate suggesting that a VEGF gradient is needed for directional growth and cartilage invasion by metaphyseal blood vessels [107]. Inhibition of this protein leads to near complete suppression of vessel invasion in mice and primates, while the restoration of bone growth occurs when inhibition is removed. VEGF is expressed in endothelial cells and in a variety of inflammatory cells such as platelets, neutrophils, eosinophils, basophils, lymphocytes, macrophages and mast cells, and is involved in wound healing [108]. Following tissue injury, endothelial cells up-regulate VEGF expression which results in increased vascular permeability and hydrostatic pressure, vasodilation and finally extravasation of inflammatory cells to the site of injury [108-111].

1.4.1 Tumour Angiogenesis

Despite these and other important functions in normal physiology, VEGF has assumed considerable importance for its role in tumour angiogenesis. Growing tumours will often develop regions of hypoxia resulting from decreased blood supply from the host vasculature and nutrient delivery to tumour cells [112-114]. Subsequently, apoptosis may occur in a proportion of cells, leaving behind those that can sustain the microenvironment of low-oxygen tension [114, 115]. These hypoxia-resistant cells produce

Hypoxia Inducible Factor-1 α (HIF-1α) which directly up-regulates VEGF expression [115]. VEGF receptors, predominantly VEGFR-2 (flk/KDR) on endothelial cells, bind VEGF which ultimately leads to the secretion of proteolytic enzymes such as urokinase plasminogen activator, heparinase and matrix metalloproteinases [106, 116]. These proteins degrade both the basement membrane and extracellular matrix via destruction of collagen and fibronectin, leading to a "leaky" vasculature and providing a scaffold for migrating endothelial cells [114, 117]. Proliferation of endothelial cells and their organization into hollow tubes, a process known as canalization, is supported by interactions between cell-associated surface proteins and the extracellular matrix [114]. The creation of a new basement membrane ensues leaving behind newly formed, tortuous, hyper-permeable blood vessels that increase the supply of oxygen and nutrients to the tumour [114, 118]. VEGF expression is maintained by cytokines (IL-1, IL-6), growth factors (TGF-α, TGF-β, Platelet-Derived Growth Factor (PDGF)), inactivation of tumour suppressor genes such as p53 and oncogenic activation of KRAS [106, 116, 119].

1.4.2 VEGF in Colorectal Cancer

In colorectal cancer, expression of VEGF has been linked to tumour cell proliferation, increased risk of liver metastasis and poorer survival time [60, 120, 121]. Immunohistochemical expression of VEGF is absent in normal colorectal mucosa, but highly immunoreactive in carcinomas [121-123]. Wong *et al.* investigated the temporal relationship between VEGF expression and tumour progression from adenoma to carcinoma [123]. They found that activation of VEGF was an early event in the adenoma-carcinoma sequence, suggesting that the "angiogenic-switch" described by Folkman may

occur in the early-phase of colorectal tumour development [105, 123]. No difference in stage-specific VEGF expression has yet been established. Evidence suggests that VEGF confers a survival advantage on tumour cells by up-regulating the anti-apoptotic protein Bcl-2 [124, 125]. A correlation between VEGF, Bcl-2 and p53 mutation has also been reported [48, 60, 121]. Microvessel density which is considered to be the most important prognostic factor in patients with Stage III colorectal cancer, is strongly associated with VEGF expression [48, 120]

The most important modifier of the biologic effect of ionizing radiation is the presence of molecular oxygen [113]. The sensitivity of cells to radiation is largely a function of the oxygen tension at the time of irradiation. Greater doses are required for equivalent cell killing under hypoxic, compared to normoxic, conditions [113]. VEGF is activated by hypoxia, thereby implicating this protein in the process of tumour response to ionizing radiation. Though the exact mechanism of the oxygen effect has not yet been determined, it is hypothesized that oxygen prolongs the half-life of free radicals generated by the interaction of radiation with water [113]. Irradiation has been reported to up-regulate VEGF mRNA levels [126]. Over-expression of VEGF following pre-operative radiotherapy has also been demonstrated in a small number of rectal cancers [127]. Anti-VEGF treatment in an animal model has been shown to increase radio-sensitivity under both normoxic and hypoxic conditions [128].

1.5 Immunohistochemistry (IHC)

IHC refers to the process of localizing proteins in cells of a tissue section, exploiting the principle of antigens in tissue binding to their respective antibodies [129]. Though initially used in surgical pathology for diagnosis and classification of tumours, IHC is now applied to the identification of potential prognostic or predictive markers in a variety of tumour types including colorectal cancer [130].

A number of tumour markers involved in processes mediating the response of rectal tumours to pre-operative radiotherapy such as apoptosis and tumour cell proliferation have been assessed by IHC. Unfortunately, this has often produced inconclusive or conflicting results [41, 45, 53-55, 61, 68-70, 74, 78-80, 121, 131-134]. Several factors may be contributing to these discrepancies including differences in fixation methods, laboratory protocols and the storage time of tissue samples [130, 135, 136]. Moreover, the lack of standardized scoring systems to evaluate the extent of immunoreactivity in tissues is recognized as an important limitation of the full potential for IHC. The cut-off scores for defining tumour "positivity" for a particular protein are often inconsistent across similar studies and are frequently selected based on ease of interpretation [41, 45, 68, 121]. Despite concerns regarding its subjective nature and reproducibility, staining intensity is often incorporated into a variety of scoring systems [135, 137]. The choice of scoring method, in particular the selection of cut-off scores for positivity, is rarely addressed, but may have a significant impact on the clinical utility of immunohistochemical findings [130, 136]. In this thesis, a methodology for determining relevant cut-off scores is proposed and validated.

1.6 Receiver Operating Characteristic (ROC) Curves

ROC curves have been frequently applied in the clinical oncology setting to evaluate and compare the sensitivity and specificity of diagnostic tests [138-143]. Moreover, the threshold value above which a test result should be considered positive for some outcome can be determined using ROC curve analysis[138]. The performance of standard and novel multi-marker models for the prediction of response in tamoxifen-treated breast cancer patients [144], the accuracy of a serum marker to correctly diagnose recurrence of colorectal cancer [145] and the efficiency of three different imaging modalities to identify local invasion in patients with rectal cancer are all examples of the use of ROC curves in clinical oncology [146].

The same principle could be applied to the selection of relevant cut-off scores for protein expression derived from IHC. First, protein expression should be scored semi-quantitatively by evaluating the proportion of immunoreactive tumour cells over the total number of tumour cells. Secondly, the sensitivity and specificity for some dichotomous outcome, such as tumour response (response versus no response) is assessed at every score. The ROC curve is generated by plotting the sensitivity on the ordinate and (1-specificity) on the abscissa. Finally, the point on the curve minimizing the trade-off between sensitivity and specificity can be selected as a relevant cut-off score above which "positive" expression is assigned. This score also corresponds to the point on the curve with the shortest distance to the point (0.0, 1.0) which theoretically has the maximum sensitivity and specificity for the outcome of interest. In order to determine the reliability of the selected cut-off scores, a re-sampling method known as bootstrapping

can be performed [147]. With bootstrapping, a certain number of equally sized re-samples, usually 100 or 1000, are created from the complete dataset. For each re-sample, ROC curve analysis is performed and a cut-off score is obtained. The distribution of cut-off scores can be evaluated and the most frequently obtained score is then selected to determine positivity.

The application of ROC curves in the context of IHC is based on the premise that the semi-quantitative assessment of scores is reproducible between pathologists. Therefore the inter-observer agreement must be confirmed prior to use. Inter-observer variation is rarely addressed, particularly in colorectal cancer, despite recognition that this is a key area of potential inaccuracy. Kirkegaard *et al.* [148] proposed the intra-class correlation coefficient (ICC) as a measure of the amount of variation in scores evaluated in a semi-quantitative manner. The ICC is defined as the ratio of the between-subject variance over the (between-subject + within-subject variances) [149, 150]. If the scores from different pathologists are considered reproducible, it may be more accurate to base ROC curve analysis on the average of these scores, in order to obtain a more precise estimate of the "true" percentage of immunoreactivity.

1.7 Predictive Modeling of Tumour Response

The majority of studies in colorectal cancer that investigate predictive markers of tumour response to pre-operative radiotherapy use a "magic-bullet" approach where only one marker at a time is evaluated. Although focusing on a single potential predictive marker may provide important information on the association of the protein with tumour

response, a multi-marker approach could result in greater sensitivity (and specificity) for the outcome thereby producing more clinically meaningful results. The differential gene and protein expression profiles of rectal cancers following irradiation underline the heterogeneity of this disease which should be reflected in the predictive models used to assess tumour response [151, 152]. Binary outcomes such as response versus no response to pre-operative radiotherapy can be studied in multivariate analysis using classification and regression tree (CART) analysis and logistic regression analysis [145, 153-157].

1.8 Research Goals

The objective of this research project was to develop a predictive model of both complete tumour response and complete or partial tumour response to a novel form of radiotherapy, namely pre-operative high-dose rate endorectal brachytherapy (HDREB) [13, 158] by studying the immunohistochemical expression of proteins p53, Bcl-2, APAF-1, VEGF and EGFR from 104 pre-treatment rectal tumour biopsies.

In Chapter 2, preliminary work is summarized. The predictive value of VEGF scored by one pathologist is investigated on a subset of patients treated with pre-operative HDREB with complete pathologic response or no response to therapy. In addition, the issue of scoring systems is addressed. These findings were published in **Cancer 104 (11), 2517-2521, 2005**. The expression of APAF-1 and its ability to predict complete or partial tumour response to pre-operative HDREB is determined. These findings were reported in **Cancer 106 (2), 284-285, 2006**. Finally, CART analysis is carried out on 62 patients with either complete or partial tumour response versus no response to pre-operative HDREB.

The most significant tumour markers contributing to tumour response are identified and the probability of downstaging for each combination of selected markers is obtained. The results of this study can be found in **Clinical Cancer Research 11 (15), 5440-5443, 2005.**

Chapters 3 and 4 focus on the issue of scoring systems and the selection of appropriate cut-off scores for determining tumour "positivity". In Chapter 3, immunoreactivity for p53, Bcl-2, APAF-1 and VEGF was evaluated by four pathologists and the inter-observer reproducibility is reported. These results were published in **Modern Pathology 19 (9), 1236-42, 2006**. In Chapter 4, the ROC curve methodology is described and applied for the first time to select cut-off scores for tumour positivity. To illustrate the method, the expression of a novel tumour marker Receptor for Hyaluronic Acid Mediated Motility (RHAMM) [159], was evaluated using a tissue microarray of 1197 colorectal cancers and ROC curve analysis was carried out. Bootstrapped replications of the data were performed 100 times in order to assess the reliability of the cut-off scores obtained in each of the 100 re-samples. The tissue microarray was obtained from the Institute of Pathology, University Hospital of Basel, Switzerland and did not consist of tumors treated with pre-operative HDREB. The ROC curve methodology applied to IHC was recently accepted by the **Journal of Clinical Pathology**.

The reproducibility of the semi-quantitative scoring method for the protein EGFR between three pathologists on a large number of tumours using the same tissue microarray of 1197 colorectal cancers was evaluated. In order to establish whether cut-off

scores were reproducible between different pathologists, ROC curve analysis is performed using each pathologist's scores separately. Subsequently, ROC curve analysis on the average scores was applied to identify the most relevant cut-offs for EGFR positivity in colorectal cancer for several different outcomes namely T stage, N stage, tumour grade, vascular invasion and survival time. The results of this study were recently submitted to the **British Journal of Cancer**.

Finally, having demonstrated the reproducibility of both the novel scoring method and ROC curve analysis for selecting clinically relevant cut-off scores, predictive models of complete and complete or partial tumour response to pre-operative HDREB are developed by logistic regression analysis in Chapter 5. The scores obtained for each pathologist are averaged for each protein. ROC curve analysis and bootstrapping is used on the average scores to determine relevant cut-off values for tumour positivity. Along with the patients' age, sex and tumour grade, univariate and multivariate analysis is carried out using a selection procedure to identify independent predictors of tumour response. Bootstrapping is performed to assess the reliability of the final predictive models. Lastly, the sensitivity and specificity of the models are obtained and cross-validated.

In this thesis, not only will tumour markers of response to pre-operative HDREB be identified but a novel methodology for evaluating immunoreactivity of proteins and determining cut-off scores for IHC will be proposed and validated.

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CHAPTER 2: Preliminary Analyses

VEGF as a predictive marker of rectal tumour response to pre-operative radio-therapy. Cancer 104 (11), 2517-2521, 2005

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2.1 Abstract

Background: Neo-adjuvant radiotherapy for rectal cancer may result in tumour downstaging or complete tumour regression leading to greater sphincter preservation. The identification of molecular predictive markers of tumour response to pre-operative radiotherapy would provide an additional tool for selecting patients most likely to benefit from treatment. The aim of this study was to determine whether VEGF expression in pre-irradiation tumour biopsies is a useful predictive marker of tumour response in patients with rectal cancer undergoing pre-operative radiotherapy. **Methods:** Immunohistochemistry for VEGF was performed on 59 pre-irradiation biopsies from patients with completely responsive (ypT0) or non-responsive tumours following pre-operative radiotherapy. VEGF positivity was evaluated using several scoring methods and the association between VEGF and tumour response was compared. The distribution of VEGF scores was obtained as well as the mean VEGF expression in the two response groups. **Results:** The mean VEGF expression in non-responsive tumours (NR) was significantly greater than in completely responsive tumours (CR) (p-value=0.0035). Nearly half (47%) of all CR tumours had a VEGF expression of 10% or less. Eleven tumours were negative (0% immunoreactivity) for the protein and all of these (100%) were complete responders. Fifty-two percent of the NR tumours had VEGF scores of 80% or greater. The four scoring methods used to determine the association between VEGF and tumour response each produced significant results (p-value<0.05). **Conclusions:** The results of this study indicate that VEGF assessed immuno-histochemically from pre-irradiation tumour biopsies, may be a useful marker of rectal tumour response to pre-operative radiotherapy.

2.2 Introduction

Neo-adjuvant radiotherapy is part of standard care for patients with advanced rectal cancer.[1] This treatment has been shown to improve survival and may reduce local recurrence rates versus surgery with or without post-operative radiotherapy.[2] In addition, tumour downstaging and complete tumour regression may be achieved with pre-operative radiotherapy leading to greater sphincter-preservation.[3, 4] The ability to predict tumour response from pre-irradiation biopsies may significantly improve the selection of patients for pre-operative radiotherapy.

Vascular endothelial growth factor (VEGF) is a potent mediator of tumour angiogenesis.[5] VEGF can be activated in tumour cells by several factors including oncogenes, tumour suppressor genes, cytokines (IL-1, IL-6) and hypoxia resulting in secretion of proteolytic enzymes and matrix metalloproteinases that degrade the basement membrane and extracellular matrix surrounding the tumour.[6, 7] These events ultimately lead to endothelial cell migration and the formation of a new vasculature that supports the growth of the tumour and its nutrient requirement.[8] *In situ* hybridization studies show that VEGF mRNA is significantly elevated in many human cancers and is associated with poor clinical outcome and higher aggressiveness of the tumour.[5, 9] VEGF has been shown to up-regulate the anti-apoptotic gene BCL-2 thereby acting as a survival factor for both endothelial and tumour cells.[10, 11] Activation of VEGF also leads to increased vascular permeability of tumour vessels causing them to be "leaky" and less efficient in their ability to diffuse oxygen caused by a decrease in partial oxygen pressure.[7, 12] This leaky

vasculature appears to contribute to less efficient delivery of chemotherapeutic agents to the tumour.[10, 13]

The aim of this study was to determine, from pre-irradiation tumour biopsies, the value of VEGF as a predictive marker of rectal tumour response to pre-operative radiotherapy.

2.3 Materials and Methods

Patients

Fifty nine patients with rectal adenocarcinoma were entered into the study and informed written consent was obtained from each. Clinical staging was performed via MRI and EUS. Patients were treated on a pre-operative conformal high-dose rate endorectal brachytherapy protocol followed by surgery 4-8 weeks later.[14] Radiation was delivered pre-operatively with an 8-channel endorectal catheter and high-dose rate remote after-loading system. A daily fraction of 6.5 Gy was administered over 4 consecutive days to a total of 26 Gy. Each patient was planned by CT simulation in order to obtain optimal conformal dosimetry.

Pathologic evaluation of the tumour specimen post-operatively identified 30 tumours with complete response (ypT0), and 29 with no response to radiotherapy (residual carcinoma). Patient and tumour characteristics are summarized in Table 1.

Immunohistochemistry

Pre-irradiation formalin-fixed paraffin-embedded tumour biopsies from all 82 patients were collected. Immunohistochemistry for VEGF was performed using the avidin-biotin complex (ABC) procedure, including heat-induced antigen retrieval procedures. Incubation with polyclonal anti-VEGF antibody (Santa Cruz Biotechnology, VEGF-A20, USA, 1:100) was carried out at 37° C for 1 hour. Negative controls were treated identically with primary antibody omitted. Tissue from glioblastoma was used as the positive control.

Scoring of VEGF Immunohistochemistry

Evaluation of VEGF immunoreactivity was made by two independent observers. The percentage of positive tumour cells was determined by each observer and the average of the two scores was obtained.

Several scoring systems have previously been used to evaluate VEGF positivity.[15-17] In this study, the average scores obtained by the observers were used to compare the following scoring methods: 1) Negative/positive: Negative tumour with 0% VEGF staining versus positive tumour with any degree of staining, 2) 10% cutoff: Positive tumour with more than 10% immunoreactive tumour cells, 3) 0, 1+, 2+, 3+: Tumour is negative for VEGF (0), has less than 20% positive cells (1+), has between 20% and 50% positive staining (2+) or has greater than 50% staining (3+), 4) Percentages: The actual percentage of positive tumour cell staining obtained by the observers. Assessment of

VEGF immunoreactivity from pre-irradiation tumour biopsies was performed blinded to post-operative tumour response.

Statistical Analysis

Patient and tumour characteristics were assessed by the Chi-square test. The Wilcoxon Rank Sum test was used to evaluate differences in mean VEGF expression between response groups. *P*-values <0.05 were considered statistically significant. Analysis of VEGF immunoreactivity and response was carried out by the Fisher's Exact and Chi-square tests for scoring methods 1 to 3. Logistic regression was used to test for differences in VEGF and tumour response in scoring method 4. All analyses were carried out using SAS, 8th edition (SAS Institute Inc., Cary, NC, USA).

2.4 Results

Cytoplasmic immunoreactivity ranged from 0% to 100%. The mean VEGF expression in NR tumours was 63% and was significantly greater than CR tumours (37.31%) (P-value = 0.0035). No significant association between age, gender stage or nodal status and tumour response was found.

The distribution of VEGF scores for each response group is shown in Figure 1. Nearly half (47%) of all CR tumours were found to have a VEGF expression of 10% or less. Of those, 11 tumours (79%) were negative for the protein (no VEGF expression). All NR tumours showed some degree of VEGF positivity. Fifteen of these 29 tumours (52%) had at least 80% immunoreactivity. Ten NR tumours had more than 90% VEGF expression,

whereas only 2 CR tumours (6%) were found in this region. The association between VEGF expression and tumour response produced by each of the 4 scoring systems is listed in Table 2. All methods yielded a statistically significant association between VEGF immunoreactivity and tumour response ($p<0.05$).

These results appear to indicate that tumours completely responsive to pre-operative brachytherapy most often express no or low levels of VEGF in their pre-treatment biopsies, whereas non-responsive tumours are generally highly immunoreactive.

2.5 Discussion

The identification of molecular predictive markers of tumour response to pre-operative radiotherapy would provide an additional tool for selecting patients most likely to benefit from treatment. Recently, the role of VEGF in angiogenesis and, particularly, in colorectal cancer has been investigated. Immunohistochemistry studies have shown VEGF to be absent in normal colorectal mucosa while carcinomas are highly immunoreactive.[18, 19] Wong et al investigated the temporal relationship between VEGF expression and tumour progression from adenoma to carcinoma.[18, 19] They found that activation of VEGF was an early event in the adenoma-carcinoma sequence suggesting that VEGF may be an angiogenesis-initiating factor in the early-phase of tumour development.[19] In colorectal carcinoma, no difference in stage-specific VEGF expression has yet been reported. Up-regulation of VEGF has been associated with poor prognosis in patients with colorectal cancer and linked to liver metastasis. [20, 21]

Hypoxia is a major inducer of VEGF activation which occurs primarily through the transcription of hypoxia-inducible factor 1-alpha (HIF-1α).[7] Tumour growth leads to limitations in oxygen diffusion provided by the host vasculature creating areas of hypoxia.[22] In response to this low oxygen tension, tumour cells either undergo apoptosis or begin to produce VEGF in order to induce vasculature that will in turn increase oxygen delivery to sustain their survival.[23] In addition, VEGF may activate Bcl-2, an anti-apoptotic protein.[10, 11] This may further contribute to the survival advantage of tumour cells expressing VEGF.

Our results show that low or absent VEGF in pre-irradiation rectal tumour biopsies is strongly associated with complete tumour response. A comparison of mean VEGF expression shows that non-responsive tumours are more highly immunoreactive and have a significantly greater overall VEGF expression than completely responsive tumours. Of those tumours negative for VEGF, all (100%) were completely responsive to therapy.

In this study, we further investigated whether a variety of frequently employed VEGF scoring methods affect the predictive value of the protein. The overwhelming majority of studies use a scoring method based on the 10% cutoff point.[18, 21, 23, 24] Our results demonstrate that VEGF may be predictive of tumour response to pre-operative brachytherapy regardless of the scoring system used. However, the selection of the scoring method may have a non-negligible affect on the final interpretation of the results. More research must be done in the area of scoring methods and how their interpretation may affect the predictive value of the protein.

Though most complete responders are found in the lower end of the distribution of VEGF scores including nearly ½ with 10% immunoreactivity or less, approximately 26% have more than 80% positive tumour cell staining for VEGF. One explanation for this may lie in the fact that the expression of VEGF is not sufficient for angiogenesis to occur.[7] Numerous anti-angiogenic proteins are secreted by tumour cells including endostatin, angiostatin and thrombospondins whose apoptotic action on endothelial cells counter-balances the effects of pro-angiogenic agents.[7, 25] The "switch" or imbalance of pro- and anti- angiogenic factors leading to tumour angiogenesis may not have yet occurred in these completely responsive yet highly immunoreactive tumours.[6] Similarly, non-responsive tumours with low VEGF levels may be more anti-angiogenic. If so, other mechanisms of radio-resistance may be in place in these tumours such as an imbalance of proliferation versus apoptosis, or deregulated cell-cycle arrest. It may therefore be important to study VEGF in combination with proteins that may have predictive potential such as p53, p27, Bcl-2 or cyclin D and E. [26-30]

2.6 Conclusion

In summary, the results of this study indicate that VEGF assessed immunohisto-chemically from pre-irradiation tumour biopsies, may be a useful marker in the prediction of tumour response to pre-operative radiotherapy.

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Table 1: Patient and Tumour Characteristics (N=59)

Characteristic	Female	Male
Age (years)		
Median	65.5	66.4
Maximum	91	88
Minimum	49	38
Tumour stage (%)		
cT2	5.9	2.9
cT3	94.1	91.2
cT4	0	5.9
Node status (%)		
Positive	35.29	29.41
Negative	64.71	70.59
Tumour response (%)		
Complete	20.3	30.5
No response	13.6	35.6
Total	33.9	66.1

Table 2: Comparison of scoring methods used to determine the association of VEGF and tumour response. P-values computed from * Fisher's Exact Test, # Chi-Square test, and +logistic regression.

Scoring Methods	*p*-values
(1) Presence/Negative	0.0007*
(2) 10% cutoff	0.0153*
(3) 0, 1+, 2+, 3+	0.0026#
(4) Percentages	0.0172+

Figure Legend

Figure 1:

Distribution of VEGF scores for the response groups. Complete response (CR), white; no response (NR), black.

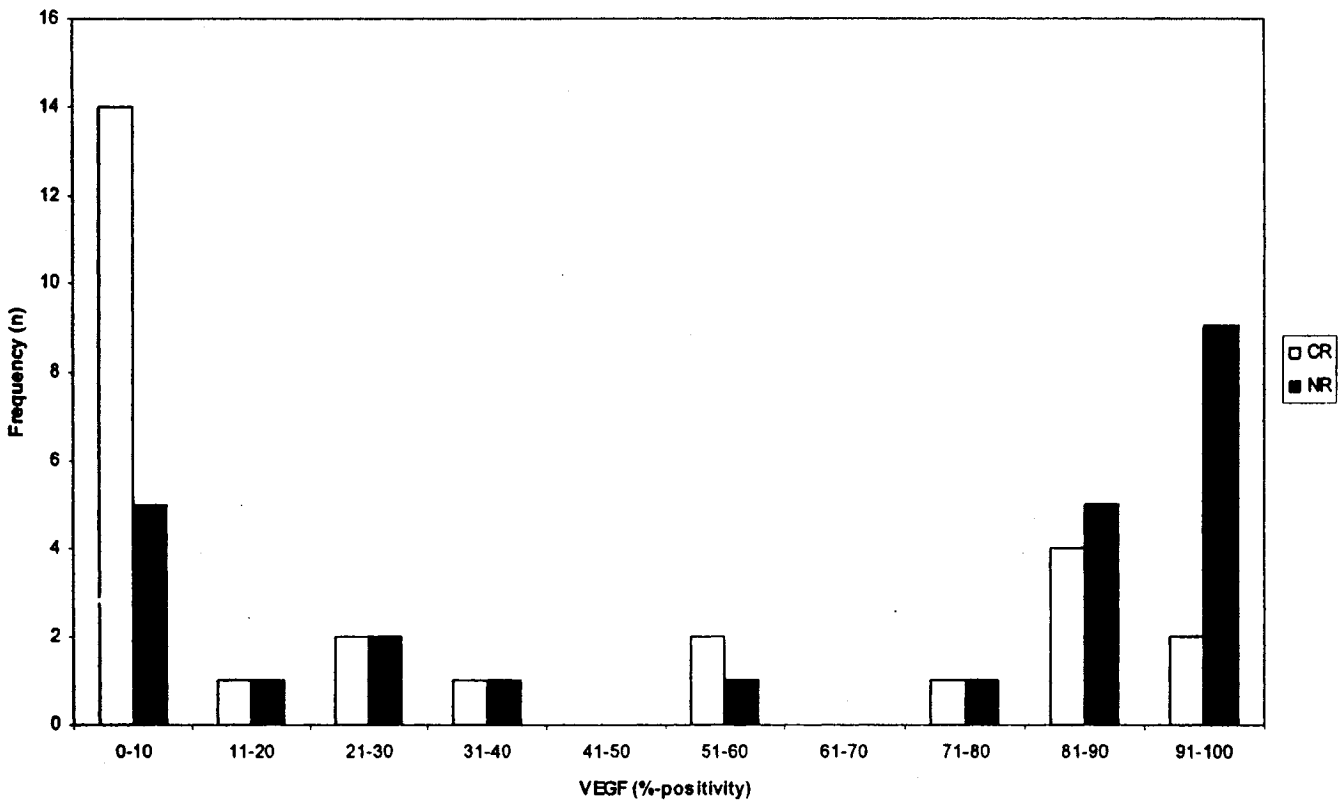
16
14
12
10
8
6
4
2
0
Frequency (n)
0-10
11-20
21-30
31-40
41-50
51-60
61-70
71-80
81-90
91-100
VEGF (%-positivity)
CR
NR

Figure 1

The predictive value of APAF-1 in rectal tumours treated with pre-operative high-dose rate brachytherapy. Cancer 106 (2), 284-285, 2006

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2.7 Abstract

Background: The aim of this study was to assess the value of APAF-1 as a predictive marker of response in rectal tumours treated with pre-operative high-dose rate endorectal brachytherapy. **Methods:** Immunohistochemistry for APAF-1 was performed on 94 rectal tumour biopsies from patients treated on a pre-operative high-dose rate brachytherapy protocol. Tumours were considered positive when more than 10% of tumour cells were immunoreactive. The association between APAF-1 expression and tumour response was made using the Chi-Square test. **Results:** Forty-four tumours (43%) were positive for APAF-1. Thirty tumours had complete pathologic tumour regression following pre-operative radiotherapy. Of these 18 were positive for APAF-1. Partial response occurred in 35 tumours. Eighteen (51%) were positive for the protein. Only 8 of the 29 (28%) non-responsive tumours were immunoreactive for APAF-1. A significant association was found between complete tumour regression and APAF-1 positivity (p-value 0.018). APAF-1 expression in partially responsive tumours was significantly greater than in non-responsive tumours (p-value 0.03). **Conclusions:** APAF-1 expression from pre-treatment rectal tumour biopsies may be a useful predictive marker of response to pre-operative radiotherapy.

2.8 Introduction

Apoptosis, or programmed cell death, is an essential process in normal development and tissue homeostasis due to the countering of abnormal cell proliferation.[1] Inhibition or deregulation of apoptotic pathways contribute to the pathogenesis of colorectal cancer and have been shown to increase tumour resistance to radiotherapy.[2] Tumour cell response to radiation may manifest itself primarily through the activation of pro-apoptotic factors resulting in mitochondria-mediated cell death.[3]

APAF-1 is a 130kD protein that plays a central role in mitochondrial apoptosis.[3] In response to apoptotic stimuli such as radiation, APAF-1 binds cytochrome c and pro-caspase 9 in the presence of ATP to form a multiproteic complex called the apoptosome.[2] Activation of pro-caspase 9 by autocatalytic cleavage initiates a cascade of downstream effector caspases ultimately resulting in apoptosis. [3, 4]

The aim of this study was to determine whether APAF-1 from pre-treatment tumour biopsies is predictive of response to pre-operative radiotherapy in patients with locally advanced rectal tumours.

2.9 Materials and Methods

Patients

Ninety-four patients with rectal adenocarcinoma were entered into the study and informed written consent was obtained from each. Patients were staged according to the International Union against Cancer classification by both endorectal ultrasonography

(EUS) and MRI. Patients with abdominal nodal disease were excluded from the study as were patients with distant metastases. Radiation was delivered pre-operatively with an 8-channel endorectal catheter using a high-dose rate remote after-loading system.[5] A daily fraction of 6.5 Gy was administered over 4 consecutive days to a total of 26 Gy. Patients were planned using a CT simulator in order to obtain optimal conformal dosimetry. The dose was prescribed to a clinical target volume that included the gross tumour volume and any intramesorectal deposits visible at MRI. Patients underwent cancer-directed surgery four-to eight weeks after brachytherapy regardless of tumour response.

Pathologic response to pre-operative radiotherapy was based on post-operative evaluation of the tumour specimen. Complete tumour response was defined as no histologic evidence of residual viable carcinoma (ypT0), partial response was determined by the presence of microfoci of residual carcinoma and non-response was characterized by large areas of residual carcinoma.

Immunohistochemistry

Immunohistochemistry was used to detect the presence of APAF-1 from each of the 94 pre-treatment tumour biopsies. Formalin-fixed, paraffin-embedded sections were cut at 3 μm and dried at 37°C overnight. Immunohistochemistry was performed using the avidin-biotin complex (ABC) procedure, including heat-induced epitope retrieval and enzymatic antigen retrieval procedures. Incubation with anti-APAF-1 (Novocastra, NCL-APAF-1, 1:100) was carried out in a moist chamber at 37 °C for 1 hour. Negative controls were treated identically with the primary antibody omitted. Positive controls consisted of

normal skin tissue. Immunohistochemistry was evaluated by two independent observers. Tumours were considered positive using the standard >10% cutoff scoring system.[6] Evaluation of APAF-1 from pre-irradiation tumour biopsies was performed blinded to post-operative tumour response.

Statistical analysis

The association of APAF-1 positivity and tumour response was carried out by the Chi-Square test. Multivariate analysis of age, sex, tumour grade and clinical stage was assessed by response. Statistics were performed using SAS Edition 8.2 (The SAS Institute, NC, USA). P-values<0.05 were considered significant.

2.10 Results

Clinical staging revealed 3 cT2, 3 cT4, and 88 cT3 tumours. Age, sex and tumour grade were not associated with tumour response. Of the 94 tumour biopsies, 43% were positive for APAF-1. Thirty tumours had complete pathologic tumour regression following pre-operative radiotherapy. Of these 18 were positive for APAF-1. Partial response occurred in 35 tumours. Eighteen (51%) were positive for the protein. Only 8 of the 29 (28%) non-responsive tumours were immunoreactive for APAF-1. A significant association was found between complete tumour regression and APAF-1 positivity (p-value 0.018). Similarly, APAF-1 expression in pre-treatment tumour biopsies from partially responsive tumours was significantly greater than in non-responsive tumours (p-value 0.03).

2.11 Discussion

Among the advantages of pre-operative radiotherapy for the treatment of locally advanced rectal cancer is tumour regression, generally carried out by rapid mitochondria-dependent apoptosis.[1] Complete pathologic tumour regression or partial tumour response can be achieved in these tumours increasing the probability of sphincter-sparing procedures.[6] The ability to predict tumour response prior to treatment using immunohistochemistry for proteins involved in programmed cell death, such as APAF-1, may provide an additional criterion for selection of patients for treatment with radiotherapy. The role of APAF-1 has been investigated in melanoma, cervical cancer and other tumour types.[7, 8] However, its value as a predictive marker in colorectal cancer has yet to be established.

APAF-1 appears to play a crucial role in normal development. APAF-1 deficient mice embryos typically die *in utero* or shortly after birth and exhibit severe craniofacial abnormalities, retention of interdigital webs, as well as abnormal eye and inner ear development.[9] APAF-1 knockouts show brain overgrowth due to hyperproliferation of neuronal cells.[10] Heterozygous mice do not show these alterations suggesting APAF-1 may function as a tumour suppressor gene.[9]

APAF-1 appears to be an essential component of p53-mediated apoptosis. Robles *et al* identified a classic p53-responsive element upstream of the APAF-1 promoter site.[11] When bound, p53 leads to the induction of APAF-1 gene expression. An inverse correlation between p53 mutation and APAF-1 expression was found in melanoma cell

lines.[11] Evidence suggests that the E2F1 transcription factor targets APAF-1 by binding at a site near the APAF-1 promoter region.[12] This activation may lead to disruption of the retinoblastoma (Rb) pathway resulting in apoptosis in a p53-independent manner.[4]

Previous studies in rectal tumours treated with pre-operative radiotherapy have investigated the potential use of apoptotic indices (the proportion of tumour cells undergoing apoptosis) from pre-treatment biopsies to predict tumour response.[13] Indices of 1% to 5% appear to correlate significantly with response whereas non-responsive tumours have a lower proportion of apoptotic tumour cells (0.5% to 1.44%).[14, 15] Though a higher apoptotic index appears to correspond to a greater likelihood of response, investigators have questioned whether assessment of apoptosis via TUNEL or H&E might not simply be a reflection of the increased proliferation rate of the tumour.[16]

The assessment of APAF-1 in rectal tumours may not necessarily be a direct reflection of the apoptotic state of the cell but rather it's potential for mitochondria-dependent cell death. Other mechanisms may be influencing APAF-1 expression. For example Bcl-2 and Bax, located between the inner and outer mitochondrial membrane, function to inhibit and stimulate cytochrome c release respectively. It may therefore, be important to study APAF-1 expression in relation to other pro- and anti-apoptotic proteins.

2.12 Conclusion

In this study, the predictive value of APAF-1 in rectal cancer was evaluated. A significant association between APAF-1 in pre-treatment rectal tumour biopsies and response to pre-

operative brachytherapy was found. We conclude that APAF-1 may be a useful predictive marker of response to pre-operative radiotherapy.

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A predictive model of rectal tumour response to pre-operative radio-therapy using classification and regression tree (CART) methods. Clinical Cancer Research 11 (15), 5440-5443, 2005

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2.13 Abstract

Purpose: The ability to predict rectal tumour response to pre-operative radiotherapy prior to treatment would significantly impact patient selection. In this study, classification and regression tree (CART) methods were used to model tumour response to pre-operative conformal high-dose rate brachytherapy by assessing the predictive value of VEGF, Bcl-2, p21, p53 and APAF-1. **Experimental Design**: Immunohistochemistry was used to detect VEGF, Bcl-2, p21, p53 and APAF-1 from 62 pre-treatment rectal tumour biopsies. Scores were assigned as percentages of positive tumour cell staining and were used in CART analysis to identify the proteins that best predicted response to radiotherapy. Ten-fold cross-validation was used to prevent over-fitting and multiple cross-validation experiments were run in order to estimate the prediction error. **Results:** Post-operative pathologic evaluation of the irradiated tumour bed revealed 43 responsive tumours (20 with complete response (T0) and 23 with partial response) and 19 non-responsive tumours. The optimal tree resulting from CART analysis had 5 terminal nodes with a misclassification rate of 18%. Of the 5 proteins selected for their predictive value, VEGF and Bcl-2 contributed most to the classification of responsive and non-responsive tumours. All 10 tumours with no VEGF were completely responsive (T0) to radiotherapy; 85% of those with VEGF and negative for Bcl-2 were responsive to therapy. **Conclusions**: VEGF and Bcl-2 status in pre-treatment rectal tumour biopsies may be predictive of response to pre-operative high-dose rate brachytherapy.

2.14 Introduction

Pre-operative radiotherapy for rectal cancer can significantly improve patient survival and reduce local recurrence rates versus post-operative radiation or surgery alone [1-4]. Additionally, high-dose rate pre-operative conformal endorectal brachytherapy, a novel therapeutic approach to the treatment of invasive rectal cancer, may result in more frequent tumour downstaging or complete tumour regression, leading to a greater number of sphincter-sparing procedures [5, 6]. The ability to predict tumour response prior to treatment may significantly impact the selection of patients for pre-operative radiotherapy as well as potentially modify post-operative treatment plans.

It is now recognized that the differential expression of genes governing cell-cycle arrest and apoptosis is an important determinant of radio-response [7, 8]. In normal cells, the p53 tumour suppressor gene mediates both cell-cycle arrest and apoptosis through the transcriptional activation of p21, BCL-2 and BAX among others [9]. In response to DNA damage, p53 enhances the transcription of p21, a cyclin-dependent kinase inhibitor that delays the progression of cells from G1 to S phase of the cell-cycle thereby preventing the replication of damaged DNA [10]. p21 has been associated with radio-sensitivity and improved outcome in rectal tumours following pre-operative radiotherapy [11-13].

Mutations of p53 in rectal cancer have been linked to decreased survival, and aggressive malignant behavior [14, 15]. Kandioler *et al* demonstrated by DNA sequencing that p53 mutations were predictive of lower survival rates and decreased response to pre-operative

radiotherapy [16]. Similar studies using immunohistochemistry to detect p53 protein yield contradicting results [17-20].

p53 may alter angiogenesis by activating vascular endothelial growth factor (VEGF), a potent mediator of new blood vessel formation in tumourigenesis [21, 22]. Expression of VEGF is induced by other factors as well, most notably hypoxia [23]. *In situ* hybridization studies have found that transcription of VEGF mRNA in rectal tumours is up-regulated during the progression from adenoma to carcinoma [21, 22, 24, 25]. Anti-VEGF therapy in combination with chemo- and/or radiotherapy for rectal cancer is an area of active investigation [23, 26].

Disruption of mitochondrial function and release of cytochrome c are early events in the apoptotic cascade [27]. In the cytoplasm, cytochrome c associates with APAF-1 initiating the downstream cleavage of caspases eventually resulting in cell death [27, 28]. Though little is known about APAF-1 function, loss or mutation of APAF-1 has been associated with radio-resistance in several tumour types [29]. Bcl-2, an anti-apoptotic protein inhibiting release of cytochrome c and activation of APAF-1, is induced by VEGF and may play a role in determining radio-response [27-29].

In this study, VEGF, Bcl-2, p21, p53 and APAF-1 in pre-treatment rectal biopsies from patients undergoing pre-operative conformal high-dose rate brachytherapy[6] were evaluated by immunohistochemistry. Classification and regression tree (CART) methods were then used to assess the value of each protein in predicting tumour response.

2.15 Materials and Methods

Patients

This study was approved by the Research Ethics Committee of the McGill University Health Center and informed written consent was obtained from sixty-two patients with rectal adenocarcinoma. Clinical staging according to the International Union against Cancer classification was carried out by both endorectal ultrasonography (EUS) and MRI. On the occasion of a disagreement between methods, the highest stage was assigned. Patients with abdominal nodal disease were excluded from the study as were patients with distant metastases. Three patients had cT2 tumours, one had cT4 and 58 were cT3. Radiation was delivered pre-operatively with an 8-channel endorectal catheter using a high-dose rate remote after-loading system. A daily fraction of 6.5 Gy was administered over 4 consecutive days to a total of 26 Gy. Each patient was planned using a CT simulator in order to obtain optimal conformal dosimetry. The dose was prescribed to a clinical target volume that included the gross tumour volume and any intramesorectal deposits visible at MRI. Patients underwent cancer-directed surgery four-to eight weeks after brachytherapy regardless of tumour response.

Tumours were classified as responsive (complete or partial response) or non-responsive to brachytherapy based on the pathologic evaluation of the specimen post-operatively. Complete response was defined as no histologic evidence of residual viable carcinoma (ypT0). Partial response was characterized by the presence of at least one micro-foci of residual carcinoma. Micro-foci ranged from 0.3 cm to 0.9 cm in diameter. Non-

responsive tumours consisted of larger areas of residual carcinoma, rather than micro-foci, that could be identified macroscopically and ranged in size from 2 cm to 6 cm.

Immunohistochemistry

Immunohistochemistry was used to detect p53, p21, Bcl-2, VEGF and APAF-1 from pre-treatment tumour biopsies. Formalin-fixed, paraffin-embedded serial sections were cut at 3 μm and dried at 37°C overnight. Immunohistochemistry was performed using the avidin-biotin complex (ABC) procedure, including heat-induced epitope retrieval and enzymatic antigen retrieval procedures. Incubation was carried out overnight at 4° C for p21 (DAKO, clone SX118, Denmark, 1:100), Bcl-2 (DAKO, clone 124, Denmark, 1:100) and VEGF (Santa Cruz Biotechnology, VEGF-A20, USA, 1:100) and in a moist chamber at 37 ° C for 1 hour for p53 (DAKO, clone DO-7, Denmark, 1:100) and APAF-1 (Novocastra, NCL-APAF-1, 1:100). Negative controls were treated identically with primary antibodies omitted. Positive controls consisted of tissue known to contain the protein of interest. Immunostaining was scored as a percentage of positive tumour cells by two independent observers.

Statistical Model

CART (Classification and Regression Tree) methods were used to determine which proteins best predicted response to treatment [30]. The CART trees were fit using the R statistical software's *tree* library package (R Foundation for Statistical Computing, 2004, Vienna Austria). The best tree fit to the full data has 8 terminal nodes (tree not shown) with an overall misclassification rate of 16% (10 out of 62).

In order to assess the amount of over-fitting, we performed 1000 10-fold cross-validation experiments [31]. In each of those 1000 experiments, the data set was randomly split into 10 smaller datasets and a pruning method was used to choose the best number of nodes for the original tree pruned with respect to 90% of the data according to the misclassification rate for the other 10% of the data. Although the best average misclassification rate across 1000 simulations was for 5 terminal nodes, the difference between 5 terminal nodes and 1 terminal node was very small (less than 1%). With further exploration, we found that average classification rate for 1 terminal node is primarily due to high variance re-sampling the small number of patients with zero traces of VEGF in the biopsy. With the reasonably large percentage of responsive tumours in the dataset, many re-sampled datasets consisted primarily of responsive tumours (which made trees with 1 terminal node competitive with 5 terminal nodes in terms of misclassification rates).

In order to resolve the uncertainty in assessing the optimal number of terminal nodes for the full data set, we conducted a two-tailed Fisher's exact test [32] to test for a relationship between the absence/presence of VEGF and response/non-response to treatment (Table 1). The p-value for the Fisher's exact test was less than 0.03, indicating a significant relationship between absence/presence of VEGF and response/non-response to treatment. Because of the instability of the full cross-validation due to the large effect of VEGF but the small number of subjects with negligible VEGF, we removed those 10 observations from the subsequent CART analyses. We fit a new classification tree with the remaining 52 observations and, using 100 10-fold cross-validation experiments, obtained an optimal

tree with 4 terminal nodes. An average cross-validated 22% misclassification rate on the 4-node sub-tree was observed conditioning on positive VEGF levels. We want to emphasize that the best number of terminal nodes for full dataset is 5 and that our sub-analysis using Fisher's exact test is merely to confirm that there is strong evidence that VEGF can be used to predict responsiveness to tumours and moderately strong evidence that the remainder of the splits in our 5-node tree can improve classification rates beyond that first split.

2.16 Results

Post-operative pathologic evaluation of the irradiated tumour bed gave rise to 43 responsive tumours (20 with complete response and 23 with partial response) and 19 non-responsive tumours. The tumour stage distribution before and after brachytherapy may be found in Table 2. Cytoplasmic immunoreactivity for VEGF, APAF-1 and Bcl-2 ranged from 0% to 100% tumour cell staining. Nuclear immunoreactivity for p53 and p21 varied from 0% to 100% and from 0% to 40% tumour cell staining respectively.

Of the 5 proteins initially selected for their potential predictive value, only VEGF, Bcl-2 and p21 contributed to the classification of responsive and non-responsive tumours (Figure 1). All ten tumours with no VEGF immunoreactivity were completely responsive to therapy (ypT0). Those with more than 2% VEGF expression were further sub-divided by the percentage of positive tumour cell staining for Bcl-2 and p21. A high classification rate was reached for tumours with no Bcl-2 and less than 92.5% immunostaining for VEGF. Such tumours were responsive to therapy in over 85% of cases whereas those

with greater VEGF levels were largely non-responsive (71%). Less efficient discrimination was observed in Bcl-2 positive tumours. Of the 10 Bcl-2-positive tumours, 8 had less than 1.5% tumour cell staining for p21.

2.17 Discussion

As tumours grow, their requirement for oxygen and nutrients expands beyond the limit of oxygen diffusion provided by the host vasculature [33]. This creates a microenvironment of hypoxia in the central region of the tumour resulting in apoptosis in cells susceptible to low oxygen tension [34]. Persistent hypoxic conditions lead to the production of VEGF [28]. This cytokine serves as a mitogen for endothelial cells and activates proteolytic enzymes involved in the degradation of the basement membrane as well as the extracellular matrix [23]. These processes ultimately result in the growth of a tumour vasculature. The new blood vessels are characterized by increased permeability rendering less efficient the delivery of chemotherapeutic agents and decreasing response to radiotherapy [23, 28]. Several studies have investigated serum VEGF levels as a prognostic marker in patients with colorectal cancer. A significant association between elevated pre-operative serum VEGF and worse prognosis has been reported [35-37].

VEGF has also been shown to act on tumour cells by inducing Bcl-2 [23, 38]. Early in the colorectal adenoma-carcinoma sequence both VEGF and Bcl-2 appear to be up-regulated [25]. In invasive cancer, VEGF levels increase whereas Bcl-2 expression may be significantly reduced [25, 39]. Bcl-2 could therefore be important primarily in sustaining cell survival under initial hypoxic conditions until oxygen and nutrients can be reached via

diffusion from newly formed tumour vessels. The presence of VEGF is likely an indirect reflection of the hypoxic state of the tumour.

Of the 10 tumours in this study that had no VEGF, all (100%) were responsive to radiotherapy. Absence of the protein may signify a well-oxygenated tumour that has not yet acquired the need for additional tumour vessels. Vascular permeability and partial oxygen pressure are maintained thereby enhancing tumour response. Bcl-2-negative tumours with low levels of VEGF may not only be retaining their vascular permeability but might also be more susceptible to radiotherapy due to a lessened anti-apoptotic signal. In this study, 85% of tumours with no Bcl-2 and with VEGF less than 92.5% were responsive to therapy. Non-responsive Bcl-2-negative tumours with nearly all cells positive for VEGF may no longer require the survival advantage of Bcl-2 provided angiogenesis has already occurred.

Several studies have described both proliferation- and apoptosis-inhibiting roles for p21 [40]. Others have reported an association between p21 in pre-treatment rectal tumour biopsies and sensitivity to pre-operative radiotherapy [12]. In our study, p21 negative/bcl-2 positive tumours were largely non-responsive to treatment (73%); p21 positive/bcl-2 negative tumours were generally associated with responsiveness (71%). However, due to the small number of tumours in our sample it may be imprudent to draw a conclusion regarding p21 from these data.

There may be several factors confounding the results of this study. First, misclassification of clinical stages using MRI for rectal cancer has recently been reported as high as 15% for pT3 tumours [41]. More than 95% of patients included in this study were staged by MRI as cT3. This may be an over-estimation of the true number of T3 tumours in our sample. The results of this study may prove to be stage-dependent. Second, protein expression in biopsies may not be representative of the entire tumour. p21 positive nuclei, for example, cluster and are typically concentrated in the upper 1/3 of the colorectal mucosa. This may possibly be contributing to the inconclusive results involving p21[42]. Thirdly, it is reasonable to assume that the time delay between pre-operative brachytherapy and surgery varies between patients. This difference may be affecting the pathologic diagnosis of response/non-response in these tumours post-operatively.

Despite these limitations, the results of this study suggest that VEGF and Bcl-2 status in pre-treatment biopsies are important in predicting response of invasive rectal tumours to pre-operative brachytherapy. Tumours absent for VEGF were associated with complete response to therapy. Those negative for Bcl-2 and with less than maximum immunoreactivity for VEGF were most frequently responsive to radiotherapy (85%).

Whether these results may be upheld across other treatment regimens such as neoadjuvant radio-chemotherapy remains to be seen. There is evidence to suggest that VEGF, Bcl-2 and p21 may play a role in predicting tumour response to this therapy [43-45]. It may however be important to tailor the selection of proteins used in the classification

and regression tree to incorporate other potential predictive markers specific to this treatment.

2.18 Conclusion

VEGF and Bcl-2 status in pre-treatment tumour biopsies may prove to be an additional tool in patient selection for pre-operative high-dose rate endorectal brachytherapy. A large-scale prospective study is necessary to validate these preliminary findings.

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Table 1: Two-way table displaying the deleterious effect of positive VEGF levels on response to treatment (p-value for Fisher's exact test of independence < 0.03).

	Response to Treatment		
VEGF Above 0	No	Yes	Total
Yes	19	33	52
No	0	10	10
Total	19	43	62

Table 2: Tumour stage distribution before and after brachytherapy

	T0	T1	T2	T3	T4	Total
Pre-treatment clinical stage (cT)	------	-----	3	58	1	62
Post-operative pathological stage (ypT)	20	11	17	14	0	62

Figure Legend:

Figure 1: Optimal tree chosen by cross-validation after preliminary step classifying by absence or presence of VEGF. The two sets of numbers underneath each terminal node are (Number of observed non-responsive subjects, Number of observed responsive subjects) and (Proportion of observed non-responsive subjects, proportion of observed responsive subjects), respectively for each terminal node.

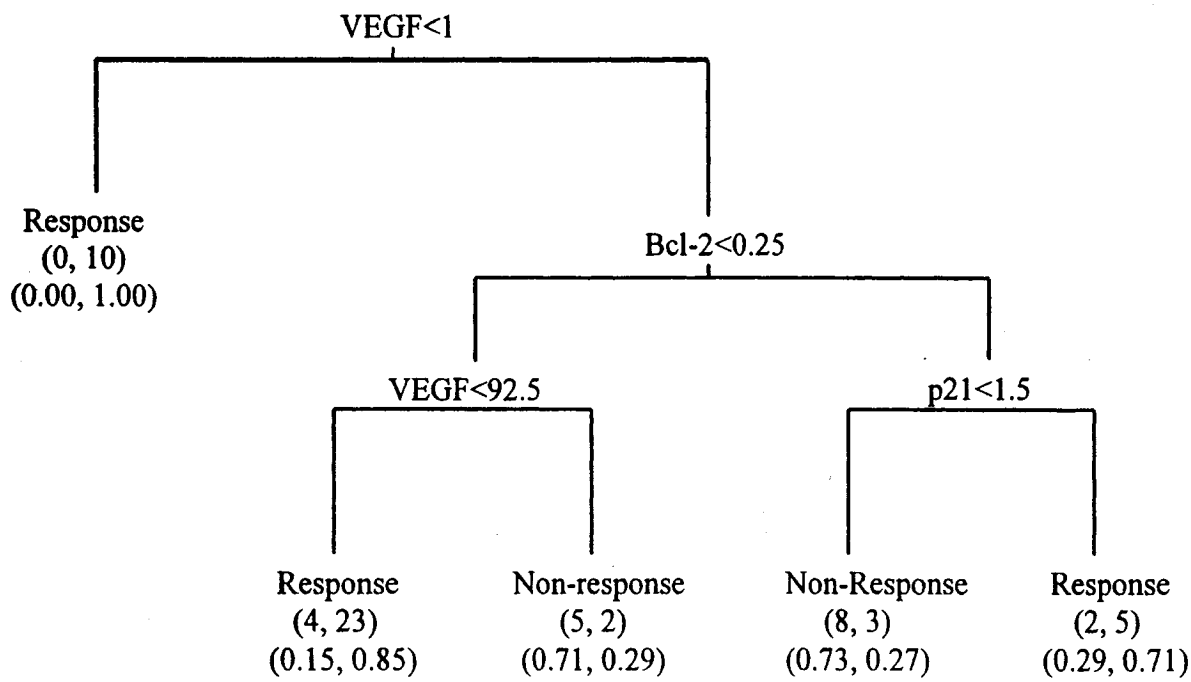
VEGF<1
Response
(0, 10)
(0.00, 1.00)
Bcl-2<0.25
VEGF<92.5
p21<1.5
Response
(4, 23)
(0.15, 0.85)
Non-response
(5, 2)
(0.71, 0.29)
Non-Response
(8, 3)
(0.73, 0.27)
Response
(2, 5)
(0.29, 0.71)

Figure 1

CHAPTER 3: Inter-observer Reproducibility of a Scoring System Based on Percentage of Positive Tumour Cells

Rather than selecting pre-determined cut-off scores to determine positivity for the proteins in Chapter 2, IHC expression was scored semi-quantitatively. This scoring method, chosen at the outset, has several advantages over the more traditional scoring systems commonly used to assess protein expression. It allows one to determine how the choice of scoring system or cut-off score influences the association of the protein and the outcome. More sophisticated statistical approaches, such as CART analysis, can be employed. Most importantly, by evaluating scores quantitatively, more clinically relevant cut-off scores for defining tumour positivity can be selected.

The value of this novel scoring method would be limited to its reproducibility between different pathologists. Therefore, in Chapter 3 the inter-observer reproducibility of the semi-quantitative scoring system is determined.

Scoring of p53, VEGF, Bcl-2 and APAF-1 immunohistochemistry and inter-observer reliability in colorectal cancer. Modern Pathology 19 (9), 1236-42, 2006

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3.1 Abstract

Aims: Molecular tumour markers are often studied in colorectal cancer using immunohistochemistry to determine their prognostic or predictive value. Protein expression is typically assigned a "positive" score based on a pre-determined cutoff. A semi-quantitative scoring method that evaluates the percentage of positive tumour cells (0%-100%) may provide a better understanding of the prognostic or predictive significance of these markers. The aim of this study was to assess and compare the inter-observer agreement of immunohistochemistry scores using a percentage scoring method and three categorical scoring systems. **Methods and Results:** Immunohistochemistry for p53, Bcl-2, vascular endothelial growth factor (VEGF) and apoptotic protease activating factor-1 (APAF-1) was performed on 87 tumour biopsies from patients with rectal carcinoma and scored independently by four pathologists as the percentage of positive tumour cells. Inter-observer agreement was assessed by the intra-class correlation coefficient. The intra-class correlation coefficients for p53 and VEGF (>0.6) indicate substantial agreement between observers. The distribution of Bcl-2 and APAF-1 scores in addition to weaker inter-observer agreement by percentage scoring suggest that this approach may not be appropriate for these proteins. **Conclusions**: p53 and VEGF protein expression assessed by immunohistochemistry in colorectal cancer and scored as a percentage of positive tumour cells may be a viable alternative scoring method.

3.2 Introduction

Although the TNM stage remains the most significant independent prognostic indicator in patients with colorectal cancer, pathologically identical tumours may neither respond to treatment uniformly nor have similar survival rates (1). A number of molecular markers involved in proliferation (p53), apoptosis (Bcl-2, APAF-1) and angiogenesis (VEGF) are currently being investigated to determine their value as prognostic or predictive factors and in turn their potential for integration into clinical practice (2,5).

Immunohistochemistry is an indispensable research and diagnostic tool used to assess the presence or absence of molecular tumour markers on paraffin-embedded tissue (6). Tumour positivity for a given marker is frequently evaluated using pre-determined cutoffs such as 10% (≤10% tumour cells staining = negative, >10% = positive) (4, 7-10). The employment of categorical scoring systems is motivated by the ease of interpretation of positive tissue by pathologists and is further supported by substantial inter-observer agreement. However, they assume that more detailed analysis of protein expression between 10% and 100%, for example will not contribute any additional relevant information in predicting outcome (11).

A semi-quantitative scoring method that assigns immunohistochemistry scores as a percentage of positive tumour cells (the number of positive tumour cells over the total number of tumour cells) may provide a more complete assessment of protein expression and a clearer understanding of the roles played by potential tumour markers in predicting outcome. Most importantly, by evaluating immunohistochemistry expression semi-

quantitatively at the outset, more relevant cutoffs for tumour positivity may be established for the protein and outcome of interest.

The greatest concern facing such a percentage scoring method is the reproducibility of the scores. In this study, we assess the inter-observer agreement of immunohistochemistry scores for 4 tumour markers known to play a role in progression of colorectal carcinoma and response to radio-therapy namely p53, VEGF, Bcl-2 and APAF-1 and compare the inter-observer agreement of percentage scoring to that of three categorical scoring systems.

3.3 Materials and Methods

Immunohistochemistry

Eighty-seven pre-treatment formalin-fixed paraffin-embedded diagnostic rectal biopsy tissues were collected from a series of patients with rectal adenocarcinoma undergoing pre-operative endorectal brachytherapy (12). Serial sections were cut at 3 μm and immunohistochemistry by the avidin-biotin complex (ABC) procedure, including heat-induced epitope retrieval, was undertaken. Incubation with the primary antibody was carried out in a moist chamber for 1 hour at 37 °C for p53 (DAKO, clone DO-7, Denmark, 1:100) and at room temperature for VEGF (Santa Cruz Biotechnology, VEGF-A20, USA, 1:100) and APAF-1 (Novocastra, NCL-APAF-1, 1:40). Overnight incubation at 4° C was performed for anti-Bcl-2 antibody (DAKO, clone 124, Denmark, 1:100). Negative controls were treated identically with the primary antibodies omitted. Positive controls consisted of tissue known to contain the protein of interest.

Evaluation of Immunohistochemistry

Nuclear positivity for p53 and cytoplasmic positivity for VEGF, Bcl-2 and APAF-1 were evaluated only in areas of invasive carcinoma. Immunoreactivity was scored as the number of positive tumour cells over total tumour cells, independently by four pathologists (CCC, JRJ, RPM, AL); in general each slide took on average 30 seconds or less to score. No specific instructions or illustrations were presented to pathologists to assist in their evaluation. Percentage scores were subsequently categorized using the 0% cutoff (0% staining versus any staining), the 10% cutoff (≤10% tumour cell staining versus >10% staining) and a three-category scoring system consisting of 0% staining, between 1% and 50% staining and >50% staining.

Statistical Analysis

The inter-observer agreement for the 0%, 10% and 0%, 1-50%, >50% cutoff scoring systems were evaluated using Light's Kappa coefficient (13). The Kappa coefficient (k) is a useful measure of agreement for categorical data as it takes into account the probability that observers achieved the same scores by chance. General guidelines for the interpretation of Kappa suggest that values between 0.81-1.0 should represent "almost perfect" agreement, 0.61-0.80 "substantial" agreement, 0.41-0.60 "moderate" agreement, 0.21-0.40 "fair" agreement, and 0-0.20 "slight" agreement (14).

The intra-class correlation coefficient is the most commonly used method to assess inter-observer agreement for quantitative measurements (15). Similar to the simple Pearson correlation coefficient that measures association, the intra-class correlation coefficient

additionally estimates agreement between scores from different observers on the same patients. The closer the intra-class correlation coefficient is to 1, the better the agreement between observers. The intra-class correlation coefficient was employed to assess inter-observer agreement of percentage scores.

Although no recommendations for the interpretation of the intra-class correlation coefficient have been detailed, reports in the literature have supported the use of the following guidelines: a coefficient of reliability >0.75 indicates "strong" agreement, between 0.4 and 0.75, "good" agreement, and <0.4, "poor" agreement (16). It has also been suggested that the values for the Kappa coefficients may be equivalent to the intra-class correlation coefficient making their direct comparison appropriate (17).

Confidence intervals (95%) were found by 10 000 bootstrap replications of the dataset. All analyses were carried out using SAS Version 8.2 (The SAS System, NC, USA).

3.4 Results

p53

Overall mean p53 protein expression was 37% (Table 1). Approximately 72% of tumours were positive for the protein. The frequency distribution of p53 scores was nearly uniform above 0% (Figure 1). The reproducibility of p53 scores was substantial for both percentage scoring and the 10% cutoff (intra-class correlation coefficient=0.755 and k=0.740 respectively) (Table 2). Excellent agreement was achieved when no positivity (0%) versus any positivity was evaluated (κ=0.831). The 0%, 1-50%, >50% scoring

method produced the least amount of agreement between observers. p53 staining was evaluated with less difficulty when no nuclei or nearly all nuclei were positive for the protein (Figure 2a). Staining intensity was generally moderate to strong. Positivity was confined to tumour cell nuclei in the majority of cases. Both the presence of cytoplasmic positivity (Figure 2b) and weak staining intensity in nuclei were largely responsible for the variation in scores.

VEGF

The distribution of VEGF scores was U-shaped (Figure 1) with an overall mean cytoplasmic expression of 45% (Table 1). The intra-class correlation coefficient for percentage scoring was 0.624 reflecting a substantial degree of inter-observer agreement (Table 2). The categorical scoring systems yielded moderate agreement between observers, the least reproducible being the 0%, 1-50%, >50% method. The intensity of staining for VEGF varied from weak to strong (Figure 2c). Considerable disagreement between scores could be attributed to weakly stained tumour cells. Infiltration of tumours with a large number of neutrophils may have contributed to the over-estimation of the number of positive tumour cells (Figure 2d).

Bcl-2

Approximately 76% of tumours demonstrated complete absence of Bcl-2 (Figure 1). Mean Bcl-2 expression was less than 10% (Table 1). Moderate inter-observer agreement was found for percentage scoring as well as for the 0% and 10% cutoffs (Table 2). Agreement was weakest for the 0%, 1-50%, >50% scoring method (k=0.407). Staining

intensity was the primary cause of disagreement of scores between pathologists. Though lymphocytes reacted strongly with the Bcl-2 antibody, only weak to moderate staining was found in tumours expressing the protein (Figure 2e). Infiltration of tumours with large numbers of lymphocytes may have also contributed to disagreement in percentage scores (Figure 2f).

APAF-1

Mean APAF-1 expression determined by each of the four pathologists varied significantly from 2.6% to 29% (Table 1). Approximately 64% of tumours were completely negative for the protein (Figure 1). Moderate agreement was achieved for percentage scoring, as well as for the 0% and 10% cutoffs. The strongest agreement was produced when no staining (0%) versus any positive staining was evaluated (k=0.514). APAF-1 positivity was strong in neutrophils and normal mucosa but only weak to moderate staining occurred in tumours expressing the protein (Figure 2g). Substantial neutrophilic infiltration in tumours may have led to disagreement between observers (Figure 2h).

3.5 Discussion

The usefulness of any immunohistochemistry scoring method is limited not only to its ability to optimize the prognostic or predictive value of tumour markers but also to its reproducibility. Studies on inter-observer agreement in colorectal carcinoma are uncommon. Several studies using the 10% cutoff scoring method describe a high degree of concordance between pathologists evaluating positive and negative tumours (18-20).

This type of agreement typically overestimates true categorical agreement by ignoring the probability that scores were obtained by chance, an important consideration when scores are not evenly distributed as was seen for Bcl-2 and APAF-1 in this study (21).

The reproducibility of p53 scores either as percentages or by way of the 10% cutoff scoring method was high. Although agreement was strongest at the 0% cutoff, the distribution of p53 expression suggests that it may be important to evaluate the complete range of scores. The inter-observer agreement of percentage scores for VEGF in this study was higher than those for the 0% and 10% cutoffs. The distribution of VEGF scores indicates that percentage scoring may provide additional information about the protein that would otherwise go unrecognized by categorizing positivity according to pre-determined cutoffs. We recently demonstrated in patients with rectal cancer undergoing pre-operative radiotherapy that mean VEGF expression was significantly higher (63%) in biopsies from patients with non-responsive tumours than from tumours with complete pathologic response (37%) (p-value=0.0035) hence exemplifying the use of percentage scores (22).

The reproducibility of Bcl-2 percentage scores was similar to the 10% cutoff. The greatest inter-observer agreement was found using the 0% cutoff. Approximately 76% of tumours in this study were completely negative for the protein. This result is in line with the literature which states that the frequency of Bcl-2 expression in rectal carcinoma is less than 30% (23). Kim *et al* demonstrated that the rate of Bcl-2 over-expression decreases with more advanced Dukes stage (23). In this study, 98% of rectal biopsies

were taken from patients with clinically diagnosed cT3 tumours. This may have biased our results in favor of the 0% cutoff and against percentage scoring as over-expression of Bcl-2 would not be expected to vary significantly in this sample. The inter-observer agreement of percentage scores may be better assessed in colorectal adenomas known to frequently over-express the protein (23). Our results show that Bcl-2 expression scored as 0% positive tumour cells versus any tumour cell staining leads to the highest degree of inter-observer agreement in rectal tumours of the same stage.

Recent evidence suggests that *APAF-1* may function as a tumour suppressor gene (24). Loss of tumour suppression leads to loss of wild-type APAF-1 protein translating into absence of staining via immunohistochemistry. It is therefore reasonable to suggest that the 0% scoring method with the highest degree of inter-observer agreement may be a more meaningful method of evaluation than scoring by percentages for this protein. Although p53 acts as a tumour suppressor gene as well a similar argument against percentage scoring cannot be used (25). The short half-life of wild-type p53 renders the protein undetectable to immunohistochemistry (26). Immunohistochemistry for mutant p53 is based on the assumption that the abnormal protein cannot act as a transcriptional factor hence accumulating in the cell (25). A comparison or DNA sequencing analysis and immunohistochemistry to detect mutant p53 has revealed a significant false-positive rate for the latter (25). Immunostaining with p53 antibodies appears therefore to detect abnormal accumulation of p53 in the cell and is not limited to detection of the mutant protein. It is possible that p53 scores evaluated as the percentage of abnormal accumulation of p53 will prove to be a useful predictive factor.

Percentage scoring should allow a more thorough assessment of the predictive or prognostic significance of tumour markers. The correlation between the immunohistochemistry expressions of several proteins can be assessed. Pich *et al* performed percentage scoring of Ki-67, PCNA and MIB-1 expression in non-Hodgkin lymphoma (27). They found a strong linear correlation for all proteins and used this finding to argue that Ki-67, PCNA and MIB-1 labeling were reliable and complementary methods to assess the proliferative activity of intermediate grade non-Hodgkin lymphoma. By studying the mean expression of Ki-67, PCNA and MIB-1, they identified sub-types of intermediate grade non-Hodgkin's lymphoma with potentially different prognoses.

Logistic regression is often used to select predictive factors from a pool of possible tumour, host or treatment variables. The risk of development of cancer using serum tumour markers (such as CEA), or the probability of local tumour control with varying doses of radiation are examples of logistic regression with quantitative variables to predict outcome (28, 29). Percentage scoring of immunohistochemistry can be applied similarly to determine how the odds of a binary outcome (response/no response to treatment) change with increases or decreases in protein expression.

Finally, by first quantifying scores, other statistical approaches such as receiver operating characteristic (ROC) analysis can be used to determine the sensitivity and specificity of tumour markers as well as the optimal cutoffs for positivity (28). By percentage scoring we have shown how classification and regression tree (CART) methods could be used to

select proteins playing a role in predicting rectal tumour response to pre-operative radiotherapy and to determine the protein cutoff values for optimal discrimination between responsive and non-responsive tumours (30).

3.6 Conclusion

Percentage scoring of immunohistochemistry expression in colorectal tumours may be suitable for proteins that exhibit a wide range of tumour cell positivity with moderate to strong staining intensity and a high degree of inter-observer agreement. The results of this preliminary study on the inter-observer agreement of percentage scoring demonstrate that the evaluation of p53 and VEGF using this approach appears to be a reproducible method and viable alternative for the evaluation of immunohistochemistry.

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Table 1: Mean and standard deviation of scores (%) for pathologists 1 to 4 and overall mean protein expression

	Overall	1	2	3	4
p53	36.90 ± 34.09	34.07 ± 33.90	34.43 ± 29.61	32.36 ± 28.67	46.71 ± 41.27
VEGF	45.15± 37.69	51. 96 ± 39.07	39.26 ± 34.43	31.11 ± 11.03	58.58 ± 39.93
Bcl-2	9.47 ± 22.98	14.16 ± 28.02	9.27 ± 22.33	4.14 ± 13.46	10.06 ± 24.48
APAF-1	17.70 ± 32.21	29.22 ± 39.27	14.85 ± 26.21	2.6 ± 7.99	23.97 ± 38.36

Table 2: Intra-class correlation coefficient measuring agreement between percentage scores and Kappa coefficients (k) measuring agreement of scores using the 0% cutoff, 10% cutoff and 0%, 1-50%, >50% cutoffs. Intervals represent 95% confidence intervals.

	N	Intra-class correlation coefficient	κ (0% cutoff)	κ (10% cutoff)	κ (0%, 1-50%, >50% cutoffs)
p53	86	0.755 (0.67, 0.82)	0.831 (0.73, 0.92)	0.740 (0.63, 0.84)	0.588 (0.48, 0.68)
VEGF	87	0.624 (0.52, 0.71)	0.565 (0.39, 0.71)	0.569 (0.45, 0.68)	0.434 (0.33, 0.53)
Bcl-2	79	0.533 (0.34, 0.69)	0.561 (0.43, 0.68)	0.49 (0.33, 0.63)	0.407 (0.26, 0.55)
APAF-1	85	0.497 (0.41, 0.58)	0.514 (0.40, 0.62)	0.434 (0.33, 0.53)	0.377 (0.30, 0.45)

Figure Legend

Figure 1: Distribution of p53, VEGF, Bcl-2 and APAF-1 scores

Figure 2: p53 (A, B), VEGF (C, D), Bcl-2 (E, F) and APAF-1 (G, H) staining. Tumours in panels A, C, E and G resulted in a high degree of inter-observer agreement whereas those in B, D, F and H lead to low inter-observer agreement.

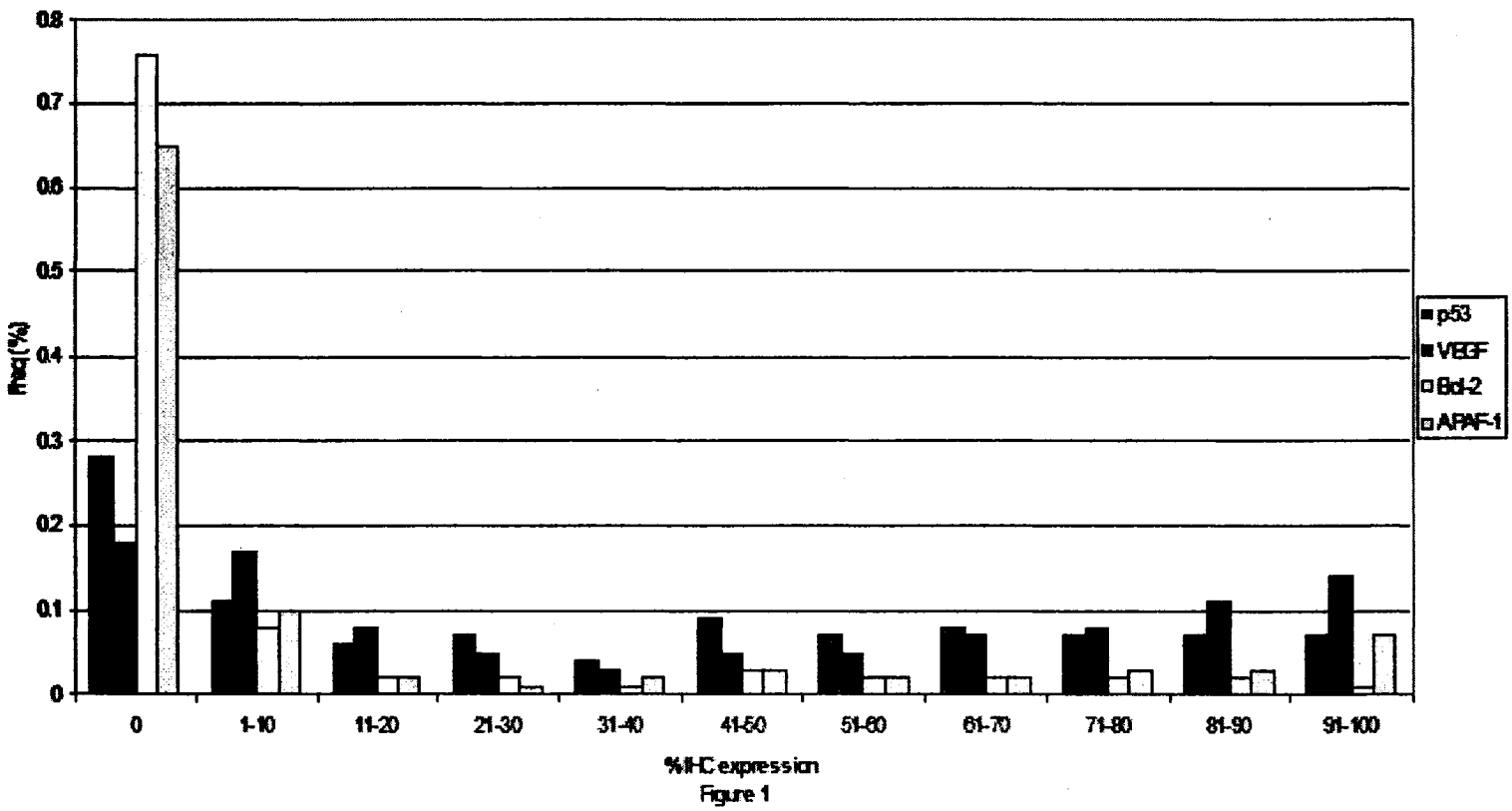
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Rhoq(%)
0
1-10
11-20
21-30
31-40
41-50
51-60
61-70
71-80
81-90
91-100
%IHC expression
p53
VEGF
Bcl-2
APAF-1

Figure 1

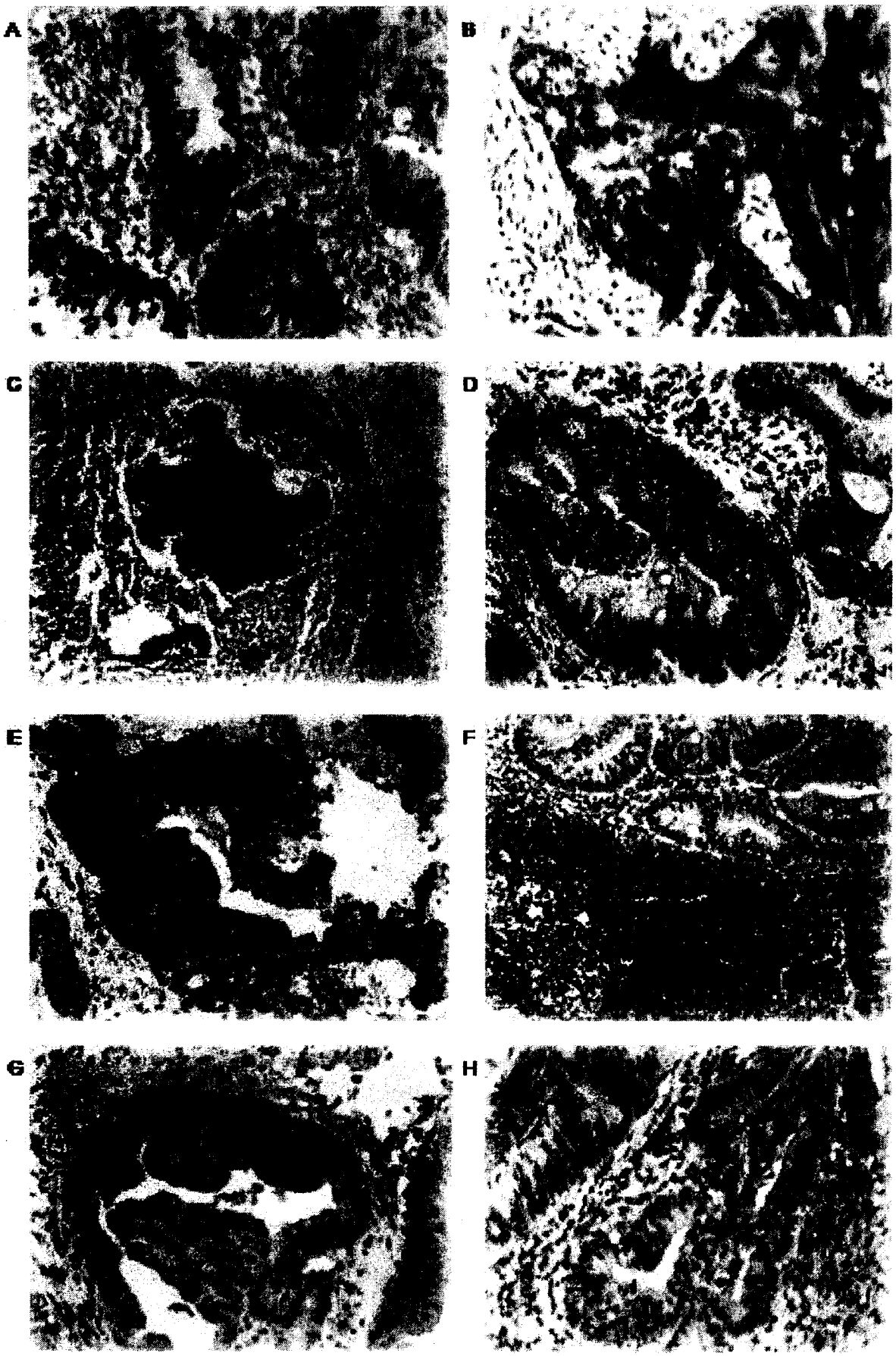

Figure 2

CHAPTER 4: Application of Receiver Operating Characteristic (ROC) Curves to the Selection of Relevant Cut-off Scores for Positivity

In Chapter 3, the semi-quantitative scoring method for evaluating IHC was found to be reproducible. The entire range of protein expression scores from 0% to 100% can therefore be analyzed using statistical approaches for quantitative data. In the following Chapter, a well-established method for determining threshold values, namely ROC curve analysis, is applied for the first time to select more clinically relevant IHC cut-off scores for defining positive protein expression. The methodology is proposed and validated on a large set of colorectal cancers with complete clinico-pathological data for the protein RHAMM. In addition, re-sampling of the data by bootstrapping is performed to determine the reproducibility of the selected cut-off scores. Finally, the consistency of selected cut-off scores between three independent pathologists is assessed for the protein EGFR using the tissue microarray (TMA) approach.

Selecting immunohistochemical cut-off scores for novel bio-markers of progression and survival in colorectal cancer. Journal of Clinical Pathology (in press)

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Key words: colorectal cancer, ROC curves, immunohistochemistry, scoring systems

4.1 Abstract

Aims: Cut-off scores for determining positivity of biomarkers detected by immunohistochemistry are often set arbitrarily and vary between reports. In the present study we evaluate the performance of receiver operating characteristic (ROC) curve analysis in determining clinically important cut-off scores for a novel tumour marker, RHAMM, and demonstrate the reproducibility of the selected cut-off scores in 967 mismatch-repair (MMR) proficient colorectal cancers (CRC). **Methods**: Immunohistochemistry for RHAMM was performed using a tissue microarray of 967 MMR-proficient CRC. Immunoreactivity was scored using a semi-quantitative scoring method by evaluating the percentage of positive tumour cells. ROC curve analysis was performed for T stage, N stage, tumour grade, vascular invasion and survival. The score with the shortest distance from the curve to the point with both maximum sensitivity and specificity, i.e., the point (0.0, 1.0), was selected as the cut-off score leading to the greatest number of tumours correctly classified as having or not having the clinical outcome. In order to determine the reliability of the selected cut-off scores, 100-bootstrapped replications were performed to re-sample the data. **Results**: The cut-off score for T stage, N stage, tumour grade and vascular invasion was 100% and that for survival 90%. The most frequently selected cut-off score from the 100 re-samples was also 100% for T stage, N stage, tumour grade, and vascular invasion and 90% for survival.

Conclusions: ROC curve analysis can be used as an alternative method in the selection and validation of cut-off scores for determining clinically relevant threshold for immunohistochemical tumour positivity.

4.2 Introduction

Immunohistochemistry (IHC) is an indispensable research tool frequently used to study tumour progression and prognosis in colorectal cancer (CRC). However, the clinical utility of its findings is largely dependent on the methods used to evaluate immunoreactivity. A large number of studies in CRC define positive protein expression using a pre-determined and often arbitrarily set cut-off score, frequently 10%[1-11]. In addition, staining intensity is often assessed despite concerns of subjectivity, reproducibility and the effect of storage time on tissue samples [12-16]. The choice of scoring method, in particular the selection of cut-off scores for positivity is rarely addressed. The lack of standardized scoring systems has led to a wide range of methods, many unvalidated, for evaluating IHC in CRC. This factor may largely be responsible for the contradictory results of similar studies evaluating the same protein and the difficulty in ascertaining the prognostic value of potential tumour markers [17].

Receiver operating characteristic (ROC) curves are commonly used in clinical oncology to evaluate and compare the sensitivity and specificity of diagnostic tests [18-23]. In addition, they allow one to identify the threshold value above which a test result should be considered positive for some outcome [18]. Established applications of ROC curve analysis in clinical oncology include the performance of standard and novel multi-marker models for the prediction of response in tamoxifen-treated breast cancer patients [24], the accuracy of carcinoembryogenic antigen to correctly diagnose recurrence of CRC compared to other serum markers [25] and the efficiency of magnetic resonance imaging

(MRI), computerized tomography (CT) and endoluminal ultrasonography (EUS) to identify local invasion in patients with rectal cancer [26].

ROC curve analysis could be applied similarly to evaluate IHC protein expression and to select biologically or clinically relevant cut-off scores for tumour positivity. We have recently demonstrated that the receptor for hyaluronic acid mediated motility (RHAMM) is an independent prognostic factor and appears to play a role in tumour progression in CRC [27]. However, RHAMM is a novel tumour marker and an established cut-off score for this protein has not previously been reported. Therefore, in the present study we evaluate the performance of ROC curve analysis in determining clinically important cut-off scores for RHAMM and demonstrate the reproducibility of the selected cut-off scores in 967 mismatch-repair (MMR) proficient CRC.

4.3 Materials and Methods

Tissue Microarray (TMA) Construction

A TMA of 1420 unselected, non-consecutive CRCs was constructed [28]. Briefly, formalin-fixed, paraffin-embedded tissue blocks of CRC resections were obtained. One tissue cylinder with a diameter of 0.6 mm was punched from morphologically representative tissue areas of each donor tissue block and brought into one recipient paraffin block (3 x 2.5 cm) using a homemade semiautomated tissue arrayer.

Clinico-pathologic data

The clinico-pathologic data for all patients included T stage (T1, T2, T3 and T4), N stage (N0, N1 and N2), tumour grade (G1, G2 and G3), vascular invasion (presence or absence) and disease-specific survival. The distribution of these features is described elsewhere [29].

IHC

Four-micron sections of TMA blocks were transferred to an adhesive-coated slide system (Instrumedics, Inc., Hackensack, NJ). Briefly, 1420 CRC punches were dewaxed and rehydrated in dH_2O. Endogenous peroxidase activity was blocked using 0.5% H_2O_2. The sections were incubated with 10% normal goat serum (Dako Cytomation, Carpinteria, CA) for 20 min and incubated with primary antibody at room temperature (MLH1 clone MLH-1, BD Biosciences Pharmingen, San Jose, CA; MSH2 clone MSH-2, BD Biosciences Pharmingen, San Jose, CA; MSH6 clone 44, Transduction Laboratories; RHAMM clone 2D6; Novocastra, UK). Subsequently, sections were incubated with peroxidase-labeled secondary antibody (DakoCytomation) for 30 min at room temperature. For visualization of the antigen, the sections were immersed in 3-amino-9-ethylcarbazole+substrate-chromogen (DakoCytomation) for 30 min, and counterstained with Gill's haematoxylin.

IHC Evaluation

Cytoplasmic immunoreactivity was scored in a semi-quantitative manner by evaluating the proportion of positive tumour cells over total tumour cells in 5% increments (0%, 5%,

10%, ..., 100%). MLH1, MSH2 and MSH6 were scored in the nucleus as negative (0%) or as positive (>0%).

MMR status

The 1420 CRCs were stratified according to DNA MMR status: (1) MMR-proficient tumours expressing MLH1, MSH2 and MSH6, (2) MLH1-negative tumours, and (3) presumed HNPCC cases demonstrating loss of MSH2 and/or MSH6 at any age, or loss of MLH1 at <55 years [30]. Only MMR-proficient tumours were included in this study (n =1197, 84.4%).

Statistical Methods

Selection of cut-off scores

The selection of clinically important cut-off scores for RHAMM expression was based on ROC curve analysis [18]. At each percentage score for RHAMM expression, the sensitivity and specificity for each outcome under study was plotted, thus generating a ROC curve. The score having the closest distance to the point with both maximum sensitivity and specificity, i.e., the point (0.0, 1.0) on the curve, was selected as the cut-off score leading to the greatest number of tumours which were correctly classified as having or not having the clinical outcome. In order to use ROC curve analysis, the clinico-pathological features were dichotomized: T stage (early (T1+T2) or late (T3+T4)), N stage (N0 (no lymph node involvement) or >N0 (any lymph node involvement)), tumour grade (low (G1 + G2) or high (G3)), vascular invasion (absent or present), and survival (death due to CRC or censored (lost to follow-up, alive or death from other causes)).

Reproducibility of cut-off scores

In order to determine the reliability of the selected cut-off scores, 100-bootstrapped replications were performed to re-sample the data[31]. With bootstrapping, 100 re-samples of equal size are created and ROC curve analysis is performed for each sub-group. The most frequently obtained cut-off score (mode) over the 100 re-samples and the area under the ROC curve (AUC) and 95% confidence interval (CI) were acquired for each analysis. The AUCs summarize the discriminatory power of RHAMM over the entire range of scores for each outcome with values of 0.5 indicating low power and those closer to 1.0 higher power. All analyses were carried out using SAS (Version 9, The SAS Institute, Cary, NC, USA).

4.4 Results

IHC

Immunoreactivity was evaluated in 967 of the 1197 MMR-proficient CRC, the discrepancy arising from lack of tissue or tumour in several TMA punches. Immunoreactivity ranged from 0% to 100%.

Selection of cut-off scores

The ROC curves for each clinico-pathological feature (Figure 1) clearly illustrate the point on the curve closest to (0.0, 1.0) which maximizes both sensitivity and specificity for the outcome. The cut-off score for T stage, N stage, tumour grade and vascular invasion was 100% and that for survival 90%.

Reproducibility of selected cut-off scores

The distribution of cut-off scores obtained from 100 re-samples of the data is shown in Figure 2. The most frequently selected cut-off score was 100% for T stage, N stage, tumour grade, and vascular invasion whereas that of survival was determined to be 90%. The AUC (95%CI) was 0.54 (0.49-0.58) for T stage, 0.56 (0.52-0.60) for N stage, 0.58 (0.52-0.65) for tumour grade and 0.54 (0.50-0.58) for vascular invasion. The AUC for survival was considerably higher at 0.69 (0.65-0.73).

4.5 Discussion

A common problem faced by researchers and pathologists involved with IHC is the determination of the extent of tumour positivity for a given marker which is clinically and biologically relevant. This is often assessed using a pre-determined cut-off score which, particularly for novel tumour markers, is often set arbitrarily and varies between different reports [1-11].

In this study we propose a method for determining cut-off scores which should improve the clinical utility of IHC findings. ROC curve analysis is an established method in other areas of medical research but has not previously been used in the context of IHC to select scores for positive protein expression [18, 19, 23, 24]. To demonstrate its application, we chose the protein RHAMM which we previously identified as a potential marker of tumour progression and prognosis in CRC [27]. However, its biological function has not been fully elucidated and so no criteria currently exist for determining biologically relevant IHC cut-off points.

The results of this study clearly demonstrate that the selected cut-off scores from ROC curve analysis are reproducible for each clinico-pathological features studied. The cut-off score leading to the best discrimination of tumours with and without the outcome was 100% (100% versus <100% staining) for T stage, N stage, tumour grade and vascular invasion and 90% (≥90% versus <90% staining) for survival.

The cut-off scores were selected such that the trade-off between sensitivity and specificity was the smallest, therefore leading to the greatest overall number of correctly classified tumours with and without the clinico-pathological feature. However, it may be more beneficial when investigating different outcomes, such as response to treatment to choose a cut-off leading to higher sensitivity rather than specificity. This would allow for the selection of the greatest number of potentially responsive candidates for treatment.

It should be emphasized that categorizing protein expression around the selected cut-off score does not imply significant statistical associations with the outcome. However, significant associations may be more biologically meaningful and more likely to occur when appropriate cut-off scores are used to assess positivity.

The use of ROC curve analysis is based on the premise that the evaluation of immunoreactivity using the percentage of positive tumour cells is a reproducible scoring method. We have previously found strong inter-observer agreement using this scoring method in several tumour markers in rectal cancer [32]. The intra-class correlation coefficient (ICC) is an accepted method for determining agreement for semi-continuous

IHC scores [33]. We have investigated the reproducibility of this scoring method on the same TMA for proteins APAF-1 and EGFR and have found the scores to be highly consistent and reproducible among pathologists (ICC = 0.75 and 0.86 respectively) (unpublished data).

It should be mentioned that time-dependent ROC curves for analyzing survival time have been established and software recently developed to analyze these outcomes (survivalROC package in R software, The R Development Core Team, Version 2.4.0, 2006) [34]. Using this method we determined that the AUC for RHAMM was 0.613 using the Kaplan-Meier estimator and 0.608 with the Nearest Neighbor Estimator. Both these results are similar to the AUC we obtained in this study. Time-dependent ROC curves are advantageous as they take into account the number of months until censoring or death from CRC. Though the classic ROC curves illustrated in this study categorize censored observations or death at the 5-year mark they are considerably simpler to use.

4.6 Conclusion

ROC curve analysis can be used as an alternative method in the selection and validation of cut-off scores for determining the most clinically relevant threshold for immunohistochemical tumour positivity. We recommend not only that this method be used for novel tumour markers but also to re-evaluate protein expression in established biomarkers that often yield contradictory results.

Acknowledgments

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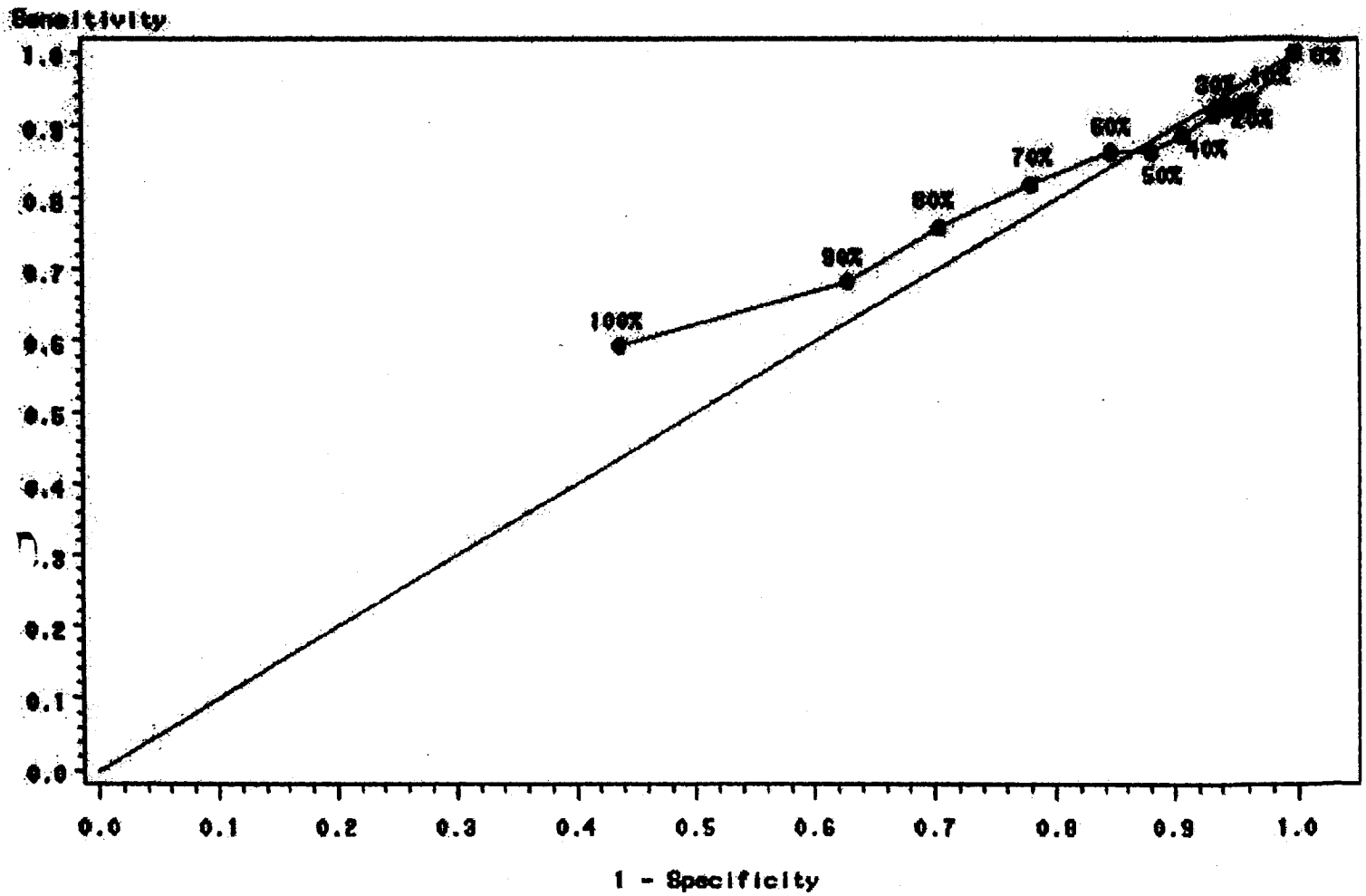
Figure Legends

Figure 1: ROC curves for RHAMM and T stage (A), N stage (B), tumour grade (C), vascular invasion (D) and survival (E). The axes for sensitivity and (1-specificity) are not equally spaced.

Figure 2: Distribution of cut-off scores obtained from 100-bootstrap replications of RHAMM.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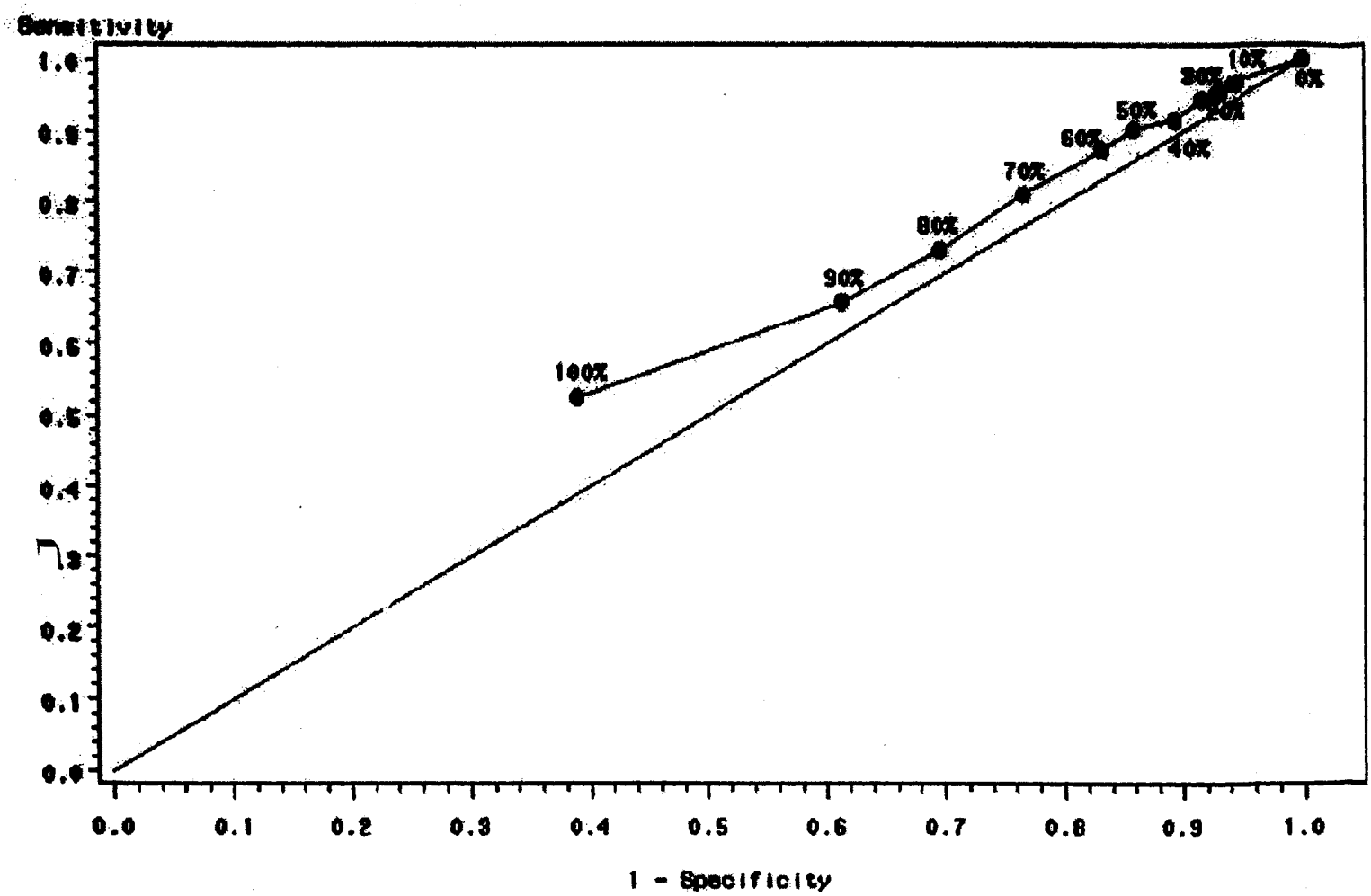
1 - Specificity

Figure 1A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1 - Specificity

Figure 1B

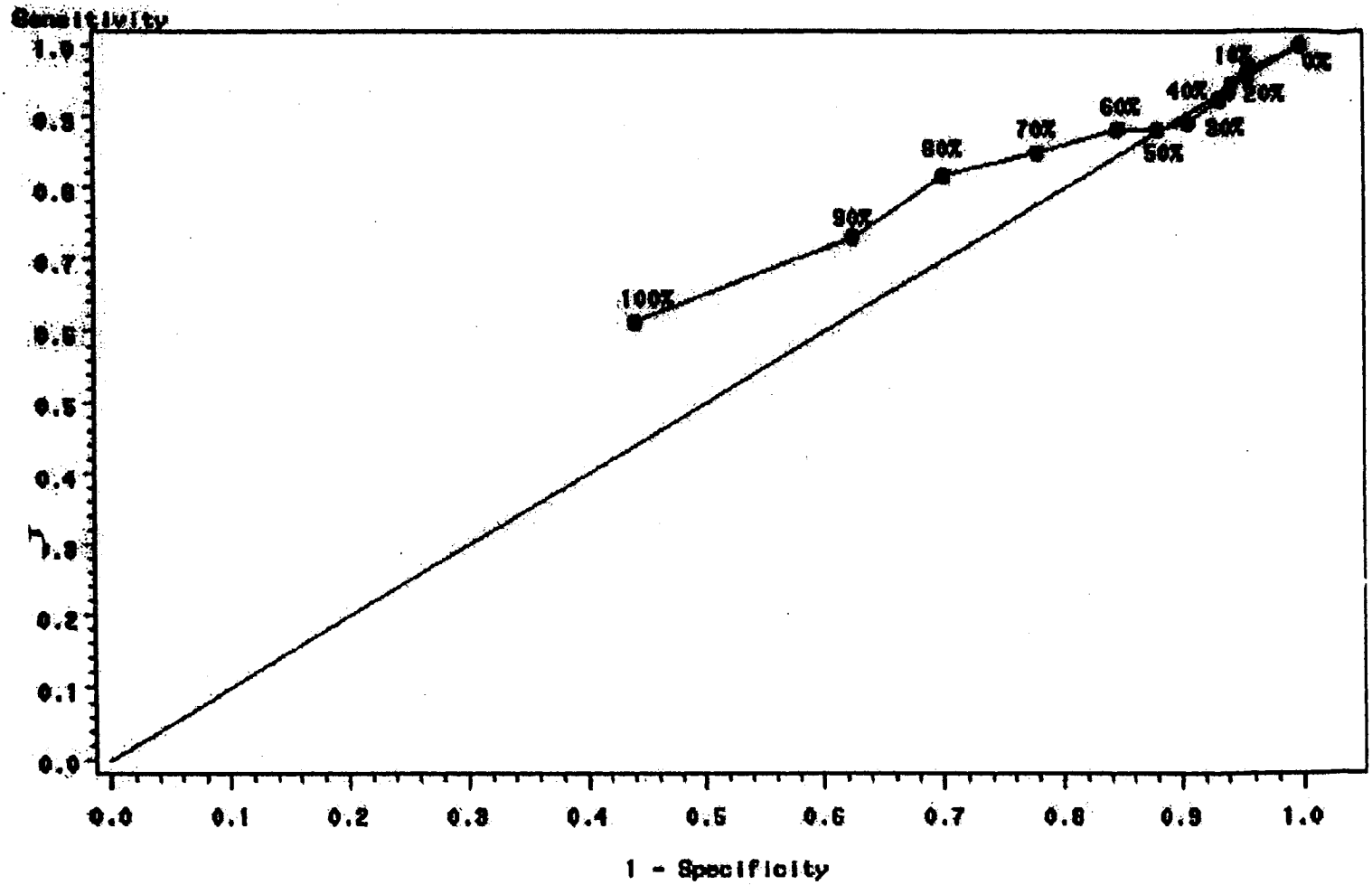
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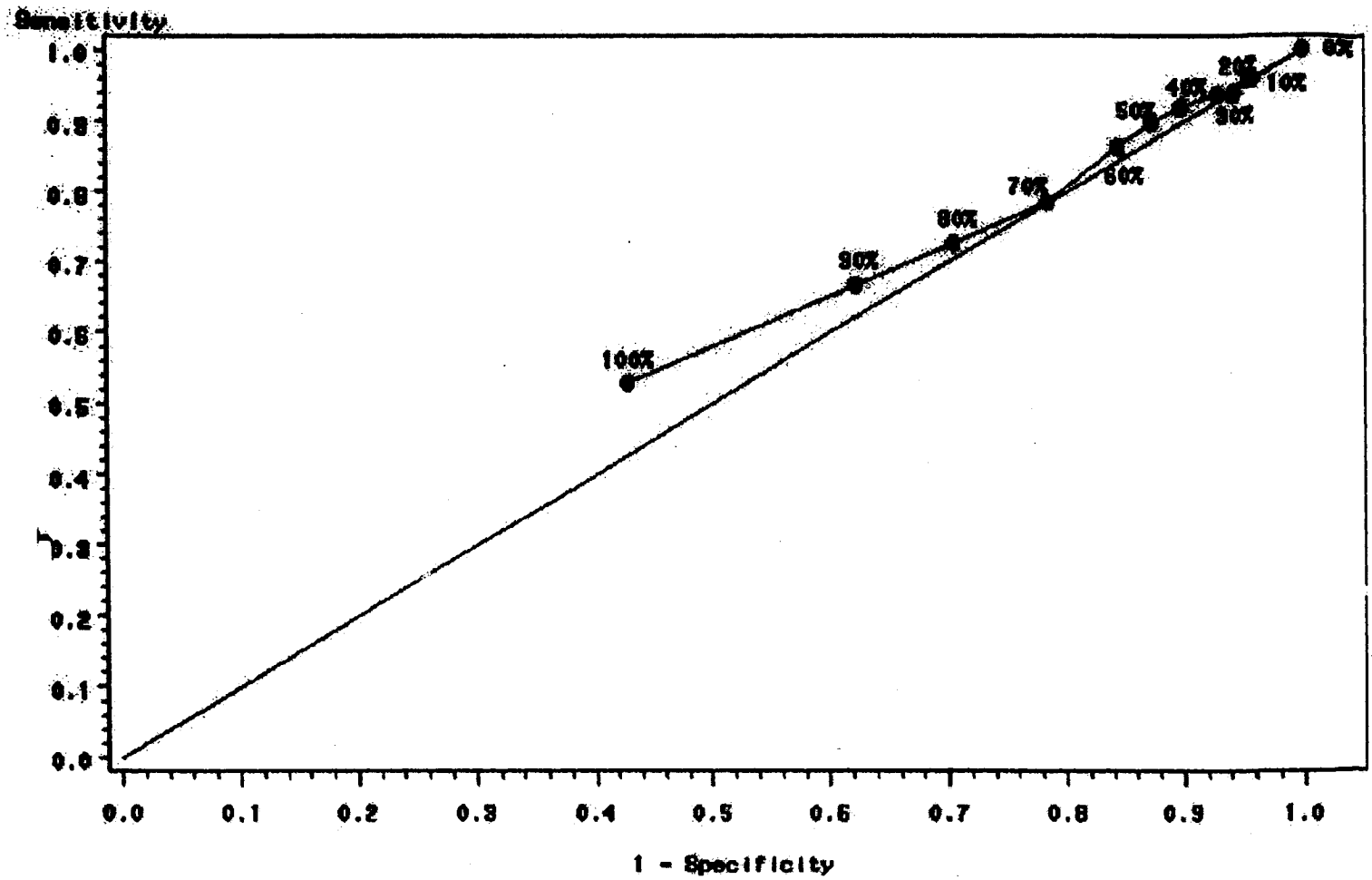
Figure 1C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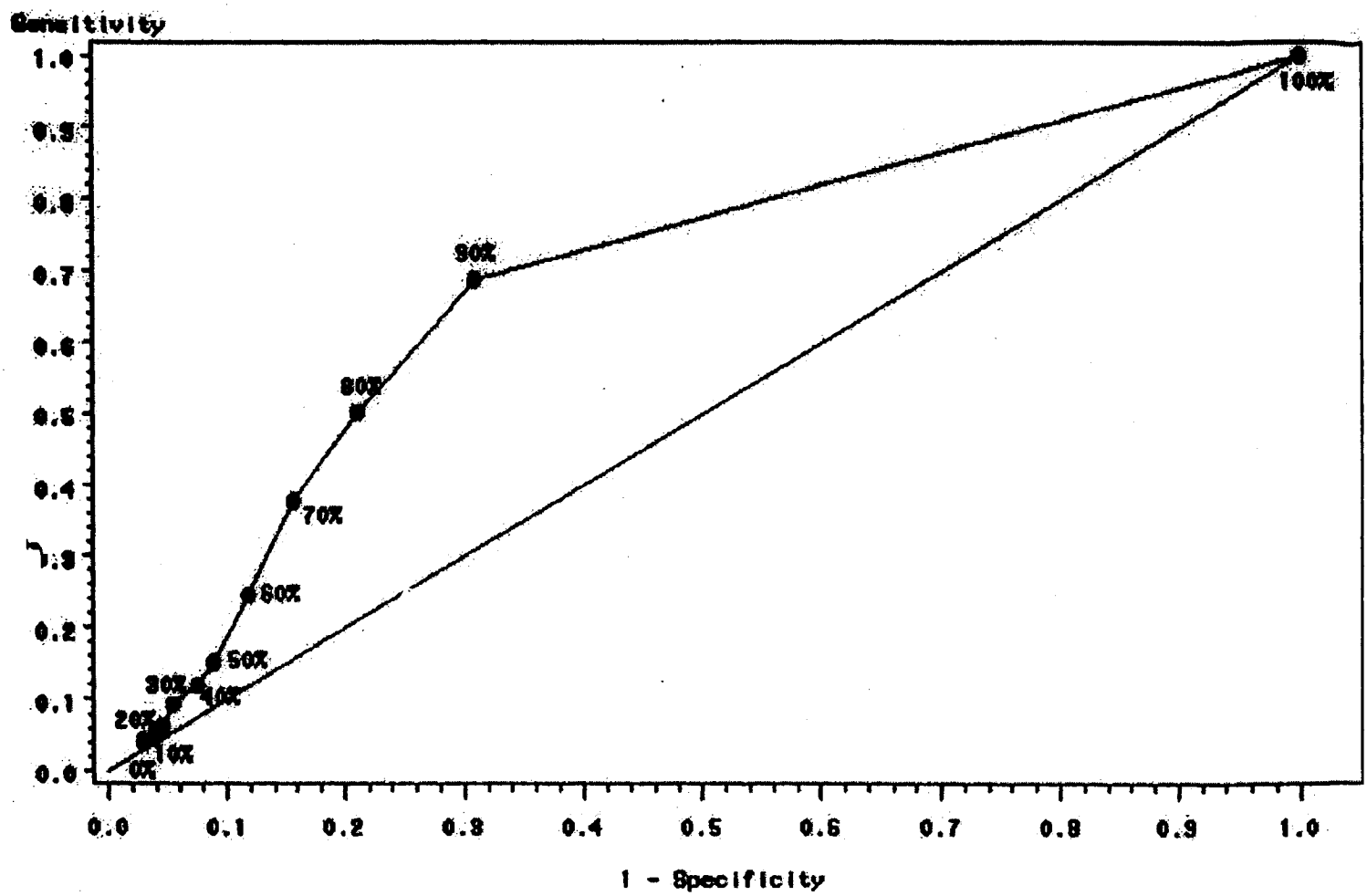
1 - Specificity

Figure 1D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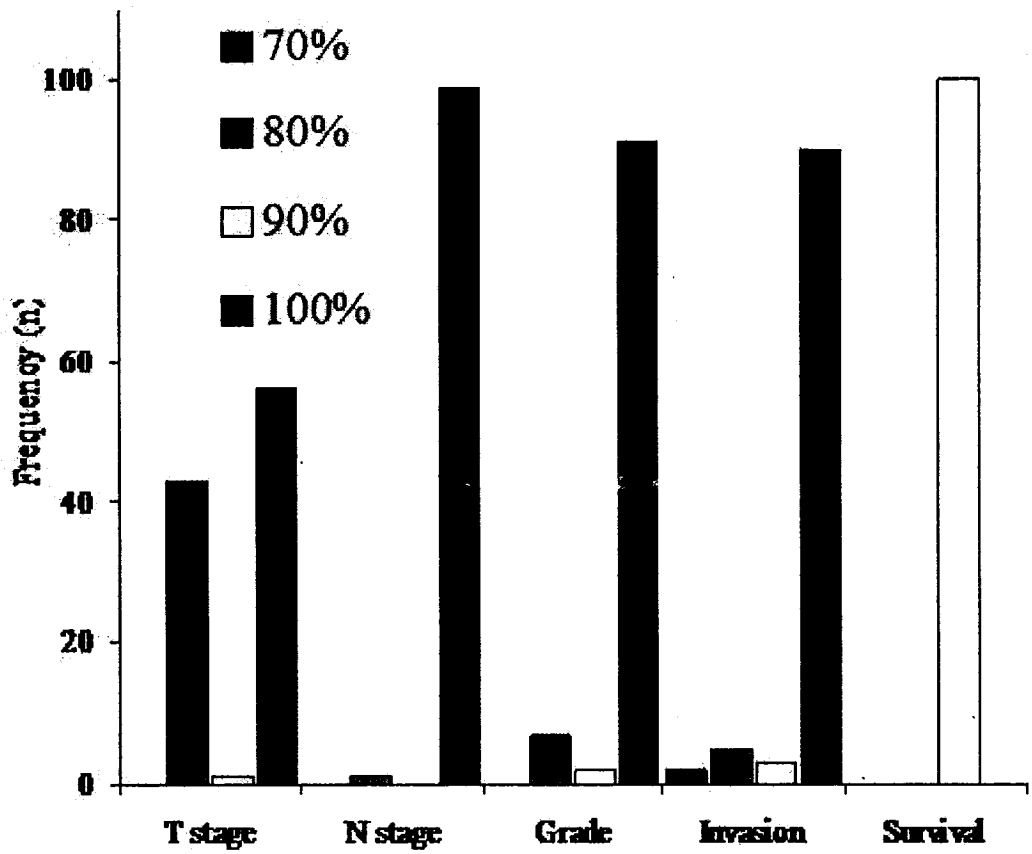
1 - Specificity

Figure 1E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Frequency (n)
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20
0
T stage
N stage
Grade
Invasion
Survival

Figure 2

A simple and reproducible scoring system for EGFR in colorectal cancer: Application to tumour progression and prognosis. British Journal of Cancer (in press)

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Running title: Scoring method for EGFR in colorectal cancer

4.7 Abstract

Aim: The aim of this study was to determine the prognostic value of epidermal growth factor receptor (EGFR) expression in mismatch-repair (MMR) proficient colorectal cancers (CRCs). We validate the use of receiver operating characteristic (ROC) curve analysis to select cut-off scores for EGFR over-expression for the endpoints studied. **Methods**: Immunohistochemistry (IHC) for EGFR was performed on 1197 MMR-proficient CRCs using a tissue microarray. Immunoreactivity was scored as the percentage of positive tumour cells by three pathologists and the inter-observer reliability was assessed. ROC curve-derived cut-offs were used to analyze the association of EGFR over-expression and several clinico-pathological features including survival. **Results**: The scoring method was found to be reproducible. The selected cut-off scores from ROC curve analysis for each clinico-pathological feature were highly consistent between pathologists. EGFR over-expression was associated with worse survival time (p-value = 0 008). In multivariate analysis EGFR over-expression was independently associated with adverse prognosis (p-value <0.001). **Conclusion**: EGFR is an independent prognostic factor in CRC.

Key Words: EGFR, colorectal cancer, ROC curve analysis, tissue microarray, scoring system

4.8 Introduction

EGFR is a 170-kDa transmembrane glycoprotein/cell surface receptor composed of an extracellular ligand-binding domain, a transmembrane lipophilic segment and an intracellular tyrosine kinase[1]. EGFR belongs to the ErbB tyrosine-kinase receptor family which includes four proteins encoded by the c-erb B proto-oncogene, namely ErbB1 (EGFR), ErbB2 (HER2/neu), ErbB3 (HER3) and ErbB4 (HER4)[2, 3]. Ligand binding produces dimerization of the receptor and activation of intrinsic protein tyrosine kinase activity leading to the transduction of signaling pathways involved in proliferation, cell division and differentiation[4]. The MAP kinase and AKT signaling pathways have been found to mediate intracellular EGFR signaling[4]. The biologic responses to MAP kinase induction result in increased expression of proteins governing cell-cycle regulation. AKT, an anti-apoptotic kinase, is implicated in cell survival and promotion of angiogenesis and has also been linked to activation of matrix metalloproteinase protein facilitating tumour growth and promotion[5, 6].

Expression of EGFR is linked to poor survival in a variety of malignancies[7-12]. In CRC, EGFR expression may be associated with an advanced disease stage[13-16]. However, these results remain controversial since an association between EGFR expression and Dukes stage or length of survival in CRC has not been detected in other studies[17-21].

Among the standard techniques for detecting protein expression, IHC is the most commonly used in CRC [22]. EGFR expression had been reported in 25% to 82% of CRCs [2, 14, 19, 23-26].

It is recognized that the wide range of methods for interpreting EGFR expression as determined by IHC considerably hinders a meta-analysis of the predictive or prognostic

value of the protein in CRC [22]. Despite its subjective nature, staining intensity has become an integral component of many EGFR scoring systems [24, 27-29]. It has recently been shown however that the degree of staining intensity may be affected by varying fixation methods and laboratory procedures and is reduced dramatically with increased storage time of the tissue samples [30, 31]. Scoring methods for EGFR include those evaluating only the degree of staining intensity [28] those for which positive or negative expression of EGFR are based on a pre-determined and often arbitrarily set cut-off score [24, 27, 32-34] and those with composite systems incorporating both the extent of positivity and staining intensity [29]. Rarely is the choice of scoring method, in particular the selection of cut-off scores for positivity, addressed and many remain unvalidated.

The aim of this study was to determine the prognostic value of EGFR in 1197 MMR-proficient CRCs using the tissue microarray (TMA) technique. In doing so, we propose and validate the application of ROC curve analysis to the selection of cut-off scores for EGFR over-expression for the endpoints under investigation.

4.9 Materials and Methods

TMA construction

A TMA of 1420 unselected, non-consecutive CRCs was constructed[35]. Briefly, formalin-fixed, paraffin-embedded tissue blocks of CRC resections were obtained. One tissue cylinder with a diameter of 0.6 mm was punched from morphologically representative tissue areas of each donor tissue block and brought into one recipient paraffin block (3 x 2.5 cm) using a homemade semiautomated tissue arrayer.

The clinico-pathological data for 1420 patients included T stage (T1, T2, T3 and T4), N stage (N0, N1 and N2), tumour grade (G1, G2 and G3), vascular invasion (presence or absence) and 5-year survival. The distribution of these features has been described previously[36].

IHC

The 1420 CRCs were dewaxed and rehydrated in dH_2O. Endogenous peroxidase activity was blocked using 0.5% H_2O_2. The sections were incubated with 10% normal goat serum (Dako Cytomation, Carpinteria, CA) for 20 min. In order to determine mismatch-repair (MMR) status, the 1420 CRCs were incubated with primary antibody for MLH1 (MLH1 clone MLH-1, BD Biosciences Pharmingen, San Jose, CA), MSH2 (clone MSH-2, BD Biosciences Pharmingen, San Jose, CA), and MSH6 (clone 44, Transduction Laboratories) for 2 hours at room temperature. Subsequently, sections were incubated with HRP-conjugated secondary antibody (K4005, EnVision+ System-HRP (AEC); DakoCytomation) for 30 min at room temperature. For visualization of the antigen, the sections were immersed in 3-amino-9-ethylcarbazole+substrate-chromogen (DakoCytomation) for 30 min, and counterstained with Gill's haematoxylin.

IHC for EGFR (clone 3C6, 3mg/ml, Ventana Medical Systems, Tucson, USA) was performed on all 1420 CRCs using an autostainer according to manufacturer's recommendations. Positive controls consisted of normal oral mucosa. Negative controls were treated identically with the primary antibody omitted.

Evaluation of IHC

EGFR immunoreactivity was evaluated as either membranous or cytoplasmic in a semi-quantitative manner using the proportion of EGFR positive tumour cells over the total number of tumour cells ranging from 0% to 100%. Scores were based on 5% intervals (0%, 5%, 10%, etc). The TMA CRCs were evaluated by 3 independent pathologists (A.L., J.J., D.H.). For the 1420 CRCs, MLH1, MSH2 and MSH6 were scored as negative (0% staining) or positive (>0% staining). Staining intensity was not evaluated.

MMR Status

The 1420 CRCs were stratified according to DNA MMR status and consisted of 1197 MMR-proficient tumours expressing MLH1, MSH2 and MSH6, 141 MLH1-negative tumours, and 82 presumed HNPCC cases demonstrating loss of MSH2 and/or MSH6 at any age, or loss of MLH1 at <55 years [37]. Only MMR-proficient tumours were included in this study to ensure a uniform population (N =1197, 84.4%).

Randomization of MMR-proficient CRCs

The 1197 MMR-proficient CRCs were randomly assigned into 2 groups, Study Group A (N=599) and Study Group B (N=598). Study Group A was used to determine the most relevant cut-off scores above which a tumour should be considered to over-express EGFR for each clinico-pathological feature. The associations of EGFR expression at the proposed cut-off scores with T stage, N stage, tumour grade, vascular invasion and survival were investigated on Study Group B.

Statistical analysis

Inter-observer reliability of the scoring method

The reproducibility of the semi-quantitative scoring method in TMA CRC punches was assessed among three pathologists and analyzed using the intra-class correlation coefficient (ICC) [38, 39]. The ICC is defined as the ratio of the between-subject variance over the (between-subject + within subject variances)[40, 41] and has previously been used to assess agreement of IHC scores [42].

Selecting the cut-off scores for EGFR "positivity"

The selection of cut-off scores for EGFR expression was based on ROC curve analysis [43]. At each score, the sensitivity and specificity for the outcome being studied was plotted thus generating a ROC curve. The score located closest to the point with both maximum sensitivity and specificity, i.e., the point (0.0, 1.0) on the curve, was selected as the cut-off score leading to the greatest number of tumours which were correctly classified as having or not having the outcome. In order to use ROC curve analysis, the clinical and tumour characteristics must be binary and were therefore dichotomized. T stage became early (T1+T2) or late (T3+T4), N stage, N0 (no lymph node involvement) or >N0 (any lymph node involvement), tumour grade low (G1 + G2) or high (G3), vascular invasion, absent or present and survival, death due to CRC at 10-year follow-up time or other (censored, alive or death from other causes).

Reproducibility of ROC curve analysis

In order to determine whether ROC curve analysis was a reproducible method for selecting the cut-off scores for EGFR, ROC curves were generated for each independent pathologist and clinico-pathological feature. In addition, 100-bootstrapped replications were performed to re-sample the data and determine the reliability of the cut-off scores obtained by each scorer. With bootstrapping, 100 re-samples of equal size are created

and ROC curve analysis is performed for each sub-group. Finally, the scores for each tumour were averaged. The final ROC curve resulting from the average scores was used to select the relevant cut-off scores and subsequently determine the association of EGFR over-expression and the clinico-pathological features on Study Group B. The most frequently obtained cut-off score (mode), the mean (95%CI) score over the 100 re-samples and the area under the ROC curve (AUC) and 95%CI were acquired for each analysis. AUCs summarize the discriminatory power of EGFR for the outcome with values of 0.5 indicating low power and those closer to 1.0 higher power.

Association with clinico-pathological features at the respective cut-offs

The Chi-Square test was used to evaluate EGFR expression with T stage, N stage, tumour grade and vascular invasion. Survival analysis was carried out using the Kaplan-Meier method and log-rank test. Cox proportional hazards regression was used in multivariate survival analysis to identify the prognostic value of EGFR independently of T stage, N stage, tumour grade, vascular invasion and age. All analyses were carried out using SAS (The SAS Institute, Cary, NC, USA). ROC curves were plotted using SPSS.

4.10 Results

Tumour characteristics

EGFR immunoreactivity was evaluated in 1032 MMR-proficient CRCs. 165 cases were not assessed due to the absence of tissue or tumour. Absence of staining was observed in 367 (35.6%) cases whereas membranous and/or cytoplasmic staining was described in 64.4% (Figure 1).

Inter-observer agreement and ROC curve analysis

The ICC obtained by analyzing the TMA CRC punches was 0.86. The characteristics of the ROC curves, the selected cut-offs generated from each pathologist's scores as well as cut-off values obtained from the average EGFR scores are summarized in Table 1. Similar cut-off scores were obtained for all three pathologists for nearly each clinico-pathological feature. The cut-off values derived from the average EGFR scores were determined to be 88.3% for T stage and tumour grade, 81.7% for N stage, 75% for vascular invasion and 91.7% for survival (Figure 2).

Association of EGFR and clinico-pathological features (Table 2)

EGFR over-expression (average score above 88.3%) was more frequently found in late T stage tumours though this difference was not significant (p-value = 0.153). No association between EGFR expression and N stage, tumour grade or vascular invasion was observed. Tumours with loss of EGFR expression (average score less than 91.2%) had a significantly better survival time (82.0 months (66.0-96.0)) (p-value = 0.008) compared to tumours retaining expression of the protein (36.5 months (20.0-65.0)) (Figure 3). In a multivariate survival analysis adjusting for T stage, N stage, tumour grade, vascular invasion and age, EGFR expression was independently associated with worse survival time (p-value <0.001 (HR (95%CI) = 2.0 (1.4-2.8)).

4.11 Discussion

The prognostic value of EGFR in CRC varies significantly in the literature. Several reasons have been suggested for this discrepancy such as non-comparable study populations[23], variability in protocols, fixation and antibodies[30] and the lack of a uniform scoring system[22, 44, 45].

The aim of this study was to determine the prognostic value of EGFR in CRC based on cut-off scores selected to maximize the clinical utility of EGFR findings by IHC. 1197 CRCs from TMA punches were randomized into two sub-groups, the first used to select the cut-off scores for EGFR over-expression, the second to analyze EGFR over-expression and its association with tumour progression and survival. The TMA approach is an accepted tool of investigation, in particular with large sample sizes[35, 46-51].

The evaluation of immunoreactivity was carried out semi-quantitatively by scoring the percentage of positive tumour cells in both rectal tumour biopsy specimens and TMA punches. We have previously shown that this scoring method leads to a more complete assessment of the prognostic value of several tumour markers in CRC when compared to an evaluation system based on arbitrarily determined "positive" or "negative" scores [36, 52-54]. We have also shown that this scoring method is reproducible among pathologists in rectal cancer using the ICC which has recently been proposed as a method for determining inter-observer variation of semi-continuous immunohistochemical scores [39, 42]. In this study we again validate this scoring method for EGFR among three independent pathologists in TMA punches of CRC (ICC = 0.86).

ROC curves are commonly used in clinical oncology to determine the threshold value above which a test result should be considered positive for some outcome [43, 55-61]. We applied the same principle in this study to determine the cut-off scores above which EGFR should be considered over-expressed. The reproducibility of this method was validated by generating ROC curves for each of the three pathologist's scores in addition to re-sampling of the data. The results of this study demonstrated that the selected cut-off scores for each clinico-pathological feature were highly consistent among pathologists.

In order to obtain the best estimate of the EGFR expression in each tumour, the three scores were averaged and the ROC curves plotted. The cut-off score varied with the clinical endpoint under investigation. EGFR was considered to be over-expressed when more than 75% staining was observed for all features.

When investigating other outcomes, such as response to anti-EGFR therapy, it may be more beneficial to choose a cut-off score leading to high sensitivity rather than specificity for tumour response in order to select the greatest number of potentially responsive candidates for treatment. In this study, the cut-off score was selected such that it minimize the trade-off between sensitivity and specificity and therefore maximize the number of correctly classified tumours with and without the endpoint under evaluation.

EGFR over-expression in MMR-proficient CRC was not associated with T stage, N stage, tumour grade or vascular invasion. These results are supported by similar findings by other groups that have shown no relationship between EGFR over-expression and disease evolution[2, 17-21, 24, 29]. However, patients with EGFR over-expressing tumours demonstrated a significantly worse prognosis (36.5 months (20.0-65.0)) than those with no over-expression (82.0 months (66.0-96.0)). Previous reports also support these findings [13, 28, 62-64]. Moreover, EGFR in this study was found to predict worse survival in a multivariate analysis independently of known adverse prognostic factors including T stage, N stage and vascular invasion. These results indicate that EGFR could be used as a prognostic marker in addition to standard pathological staging using the TNM classification.

4.12 Conclusion

In conclusion, EGFR is an independent adverse prognostic factor in MMR-proficient CRC. The combination of semi-quantitative evaluation of protein expression and ROC curve analysis which was validated in this study proves to be a reproducible method for selecting the cut-off scores for EGFR over-expression in CRC.

Acknowledgments

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Table 1: Most frequently obtained cut-off score (mode), mean (95%CI) cut-off score and area under the ROC curve (AUC (95%CI)) for each pathologist and clinico-pathological feature.

		Pathologist			
	ROC features	No. 1	No. 2	No. 3	Average scores
T stage	Mode	90%	85%	80%	88.3%
	Mean (95%CI)	83.0% (80.6-85.4)	76.5% (73.8-79.2)	77.8 %(75.4-80.2)	82.4% (79.5-85.2)
	AUC (95%CI)	0.594 (0.48-0.71)	0.579 (0.45-0.70)	0.533 (0.47-0.60)	0.609 (0.48-0.74)
N stage	Mode	80%	75%	70%	81.7%
	Mean (95%CI)	82.6% (81.5-83.7)	79.1% (77.8-80.4)	75.2% (72.9-77.4)	82.7% (81.6-83.9)
	AUC (95%CI)	0.536 (0.45-0.62)	0.552 (0.47-0.64)	0.505 (0.45-0.55)	0.553 (0.46-0.64)
Grade	Mode	90%	85%	60%	88.3%
	Mean (95%CI)	88.3% (85.8-90.8)	81.7% (78.4-85.0)	64.0% (59.7-68.3)	85.9% (83.4-88.3)
	AUC (95%CI)	0.587 (0.41-0.77)	0.574 (0.41-0.74)	0.513 (0.43-0.60)	0.586 (0.40-0.77)
Vascular invasion	Mode	90%	75%	80%	75%
	Mean (95%CI)	86.8% (85.4-88.3)	75.6% (75.0-76.2)	75.5% (72.9-78.1)	78.4 (77.3-79.5)
	AUC (95%CI)	0.548 (0.45-0.64)	0.61 (0.52-0.70)	0.515 (0.46-0.57)	0.579 (0.48-0.68)
Survival	Mode	85%	90%	80%	91.7%
	Mean (95%CI)	86.3% (85.2-87.4)	85.4% (84.2-87.1)	75.3% (72.6-78.0)	59.0% (49.2-68.8)
	AUC (95%CI)	0.523 (0.44-0.61)	0.536 (0.45-0.62)	0.501 (0.44-0.56)	0.511 (0.42-0.60)

Table 2: Association of EGFR expression and clinico-pathological features. Cut-off scores were obtained by ROC curve analysis performed on the average EGFR scores.

		Cut-off	Below cut-off N (%)	Above cut-off N (%)	P-value
Survival	Median (95%CI) (months)	91.7%	82.0 (66.0-96.0)	36.5 (20.0-65.0)	0.008
T stage	Early (T1+T2)	88.3%	119 (23.7)	13 (16.5)	0.153
	Late (T3+T4)		383 (76.3)	66 (83.5)	
N stage	N0	78.3%	245 (52.4)	53 (51.0)	0.798
	>N0		223 (47.7)	51 (49.0)	
Grade	G1+G2	88.3%	446 (88.1)	74 (93.7)	0.146
	G3		60 (11.9)	5 (6.3)	
Vascular invasion	Presence	75%	133 (27.8)	30 (28.3)	0.911
	Absence		346 (72.2)	76 (71.7)	

Figure legend

Figure 1: Predominantly membranous (A) and cytoplasmic (B) EGFR expression in rectal adenocarcinoma (40x). Membranous (C) and cytoplasmic (D) EGFR staining in TMA punches of moderately differentiated MMR-proficient CRCs (40x).

Figure 2: ROC curves based on average EGFR scores for A) T stage, B) N stage, C) grade, D) vascular invasion and E) survival. Arrows indicate the closest point on the ROC curves to the point (0.0, 1.0) which correspond to the selected cut-off score.

Figure 3: Kaplan-Meier survival curve for MMR-proficient CRCs with and without over-expression of EGFR.

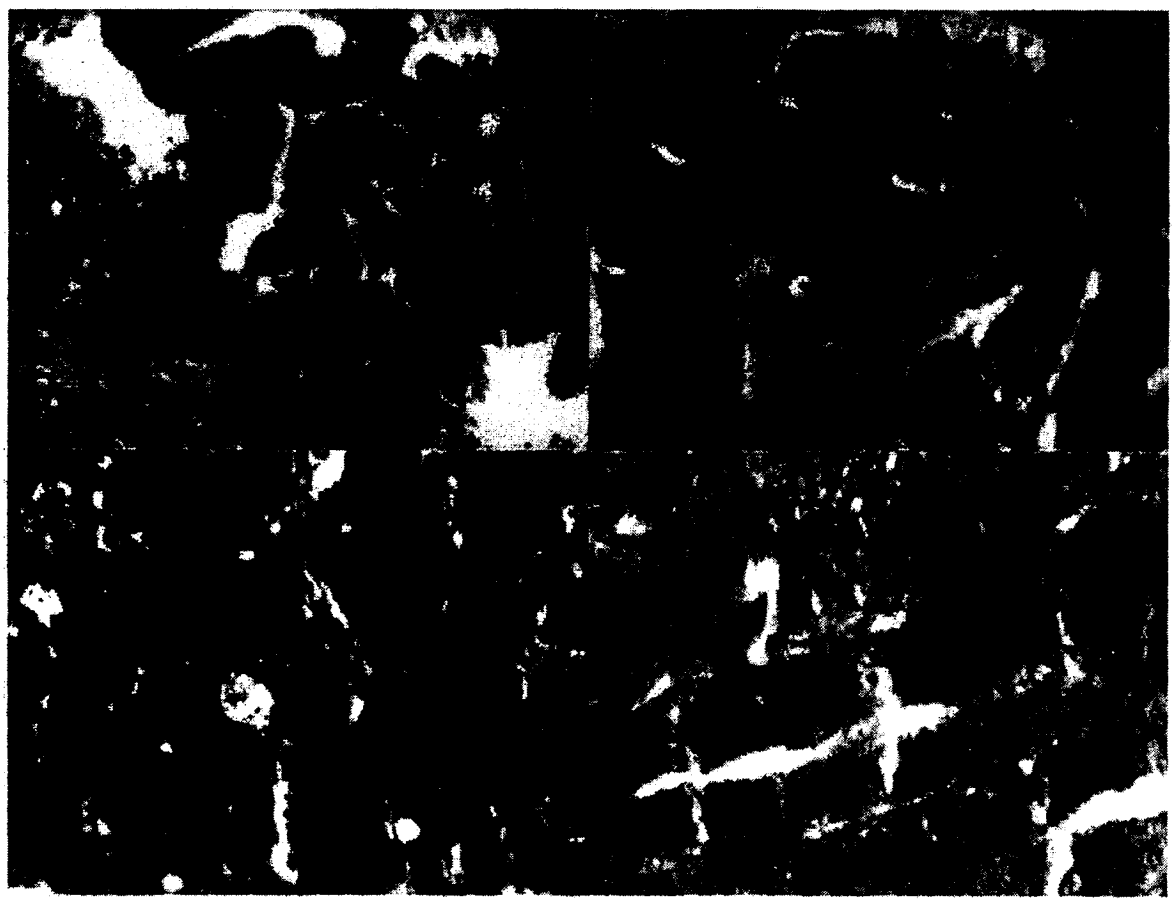
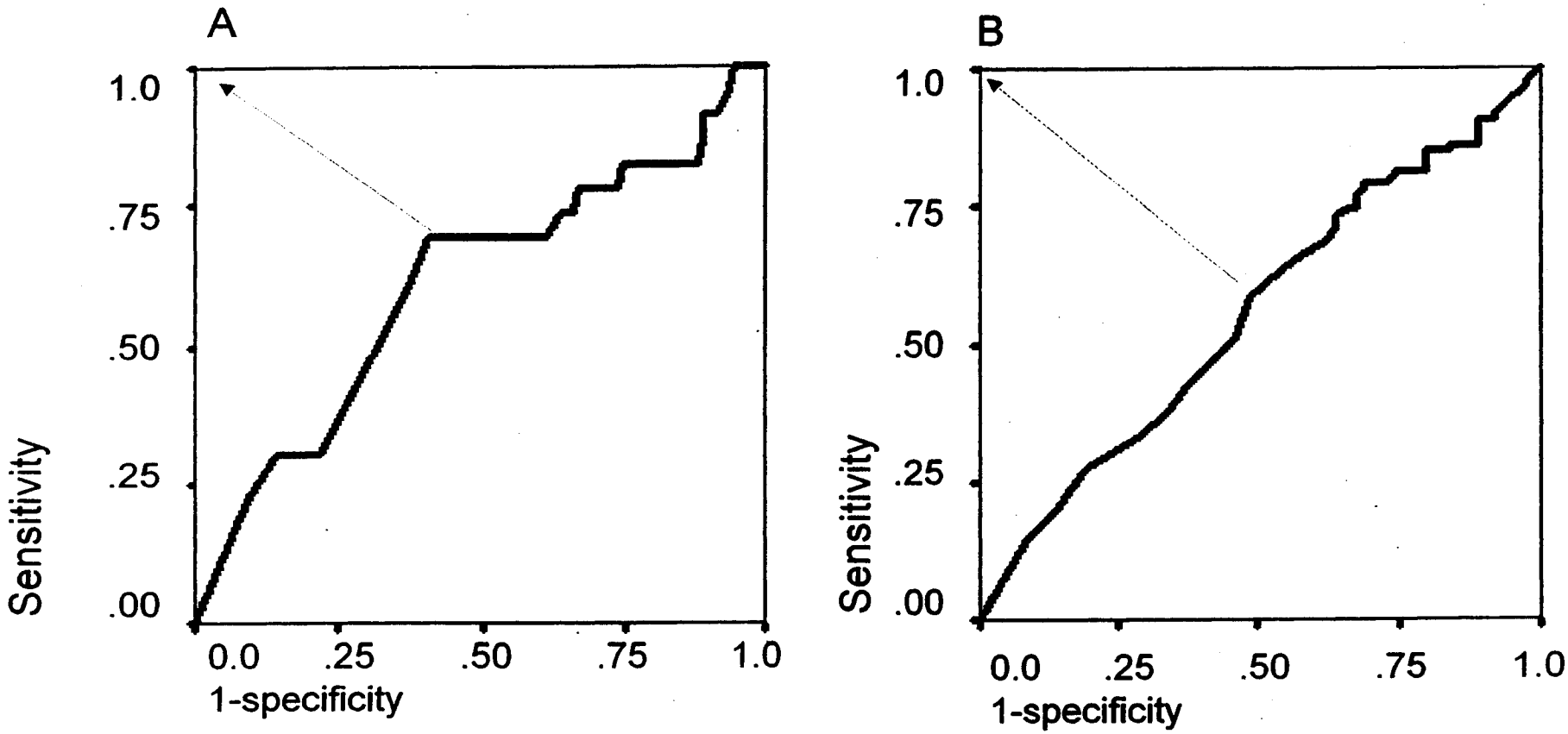


Figure 1


A
1.0
.75
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.75
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1-specificity
B
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Sensitivity
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.50
.75
1.0
1-specificity

Figure 2

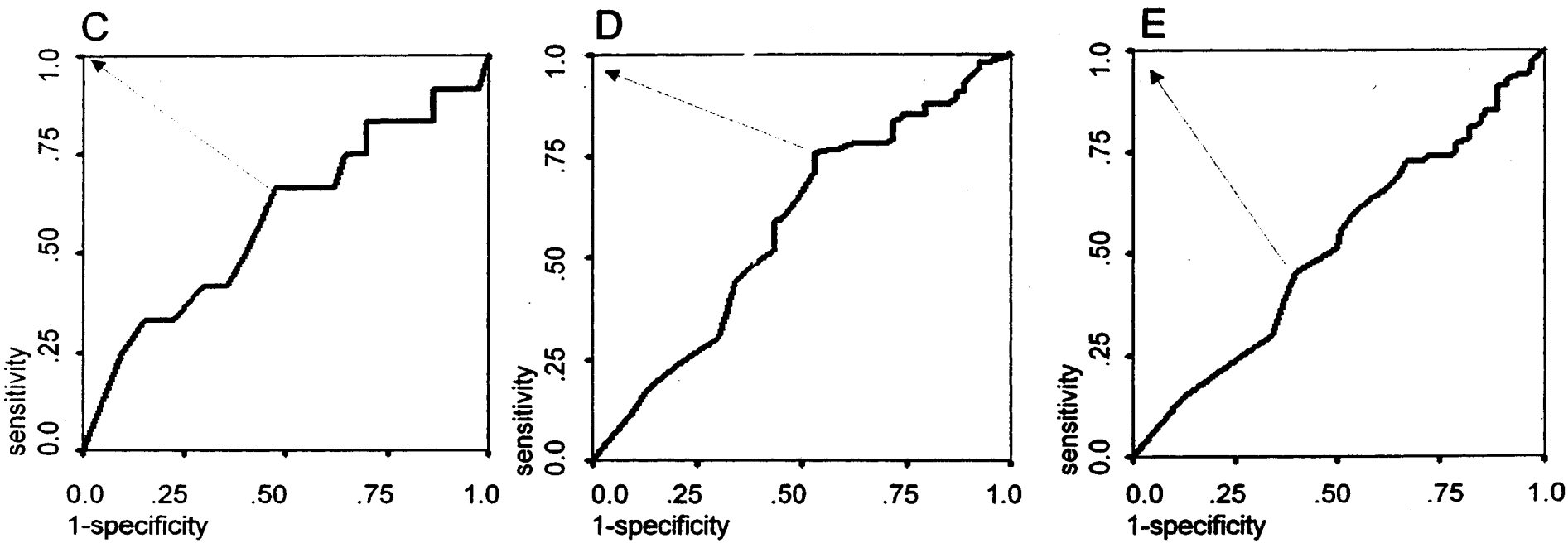

Figure 2

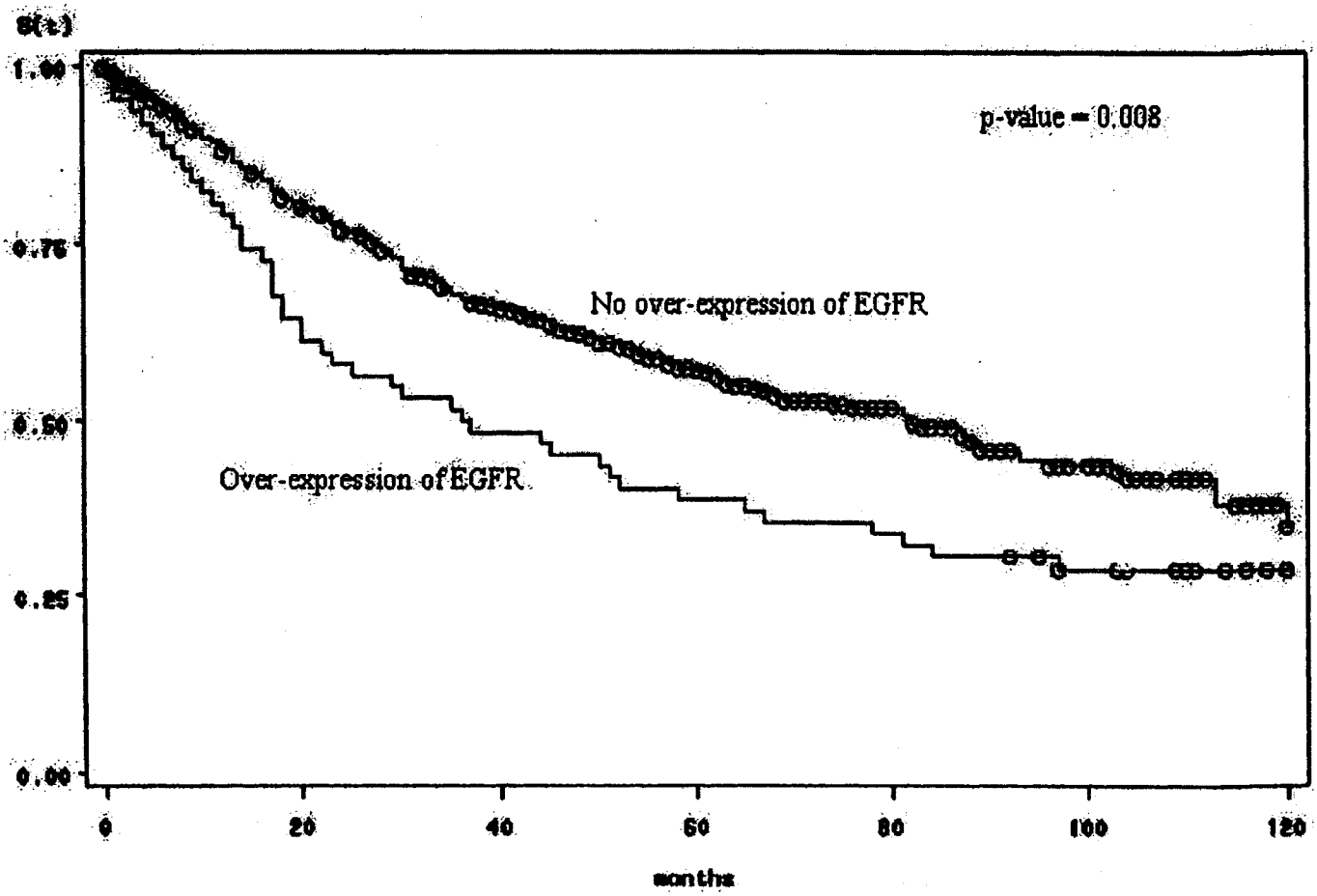
S(t)
1.00
0.75
0.50
0.25
0.00
p-value = 0.008
No over-expression of EGFR
Over-expression of EGFR
0
20
40
60
80
100
120
months

Figure 3

CHAPTER 5: Predictive Model of Tumour Response to Pre-operative HDREB

The concepts of Chapters 3 and 4 formed the basis on which the predictive model of tumour response to pre-operative HDREB was built. Having demonstrated that the semi-quantitative scoring method was reproducible between pathologists and that ROC curve analysis could be used to select cut-off scores for protein positivity, the protein expression of tumour markers p53, Bcl-2, VEGF, APAF-1 and EGFR was analyzed in pre-treatment rectal tumour biopsies from patients undergoing radiotherapy. Cut-off scores for positivity were obtained using the average protein expression for each tumour marker. Along with other clinico-pathological features, a predictive model of complete tumour response as well as a model of complete or partial tumour regression was established.

Combined analysis of VEGF and EGFR predicts complete tumour response in rectal cancer treated with pre-operative high-dose rate endorectal brachytherapy

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Running title: VEGF and EGFR predict complete response

Key words: rectal cancer, VEGF, EGFR, pre-operative radiotherapy, brachytherapy

5.1 Abstract

Aim: Pre-operative radiotherapy improves local control and survival in patients with locally advanced rectal cancer. To date, no clinically useful predictors of tumor response have been established. The aim of this study was to develop a predictive model of tumor response to pre-operative high-dose rate endorectal brachytherapy (HDREB) by evaluating the immunohistochemical expression of p53, Bcl-2, VEGF, APAF-1 and EGFR on 104 pre-treatment rectal biopsies from patients with predominantly cT3 rectal cancer. **Material and Methods**: Immunohistochemistry was performed for p53, Bcl-2, VEGF, APAF-1 and EGFR. Immunoreactivity was scored semi-quantitatively by evaluating the percentage of positive tumor cells. The reproducibility of the scoring method was assessed among three or four pathologists. Receiver operating characteristic (ROC) curves were used to obtain relevant cut-off scores for positive protein expression. Univariate and multivariate analysis was performed in order to determine the predictive value of each tumor marker. **Results**: In univariate analysis, negative VEGF expression (p-value = 0.004) and EGFR positivity (p-value = 0.003) were associated with complete tumor response whereas APAF-1 (p-value = 0.015) and EGFR positivity (p-value = 0.027) were important in complete and/or partial tumor response. In a multivariate model, the combined analysis of VEGF (p-value = 0.003) and EGFR (p = 0.006) was highly predictive of complete response with a sensitivity of 94% and specificity of 45%. EGFR independently predicted complete and/or partial response (p = 0.027) but only displayed low sensitivity (58%) for tumor response. **Conclusion**: The combined analysis of VEGF and EGFR is predictive of complete pathologic tumor response to pre-operative HDREB. A large-scale prospective study is necessary to validate these preliminary findings.

5.2 Introduction

Colorectal cancer is a leading cause of cancer-related mortality and morbidity in North America [1]. Patients with early rectal cancers are treated with local excision and may be candidates for endocavitary irradiation [2-5]. However, most patients with newly diagnosed rectal carcinoma present with locally advanced disease and receive neo-adjuvant chemo-radiation therapy. Pre-operative radiotherapy has been shown to increase survival rates and improve local control compared to surgery-alone [6-8]. Recent findings have demonstrated similar survival rates in patients receiving only neo-adjuvant radiotherapy compared to those receiving both chemo-radiotherapy [9-11]. In addition to these clinical endpoints, pre-operative chemo-radiotherapy leads to tumour regression and downstaging in a significant number of patients [12-14] potentially increasing the frequency of sphincter-sparing procedures in some studies [7, 15]. Tumour regression grade has been linked with improved disease-free survival and decreased local failure [16, 17]. Pathologic stage after treatment has also shown to have prognostic value [18].

The ability to predict complete pathologic response or sensitivity to radiation would have a significant impact on the selection of patients for pre-operative radiotherapy or chemo-radiation therapy as well as on post-surgical management. Currently there are no clinically useful predictors of tumour response based either on standard pathological assessment or on immunocytochemistry [19]. A number of tumour markers involved in proliferation (Ki-67, PCNA, p53) cell-cycle arrest (p21, p27) and apoptosis (Bcl-2) have been studied immunohistochemically but have often yielded negative or contradicting results [20-28].

Several factors may be contributing to these discrepancies most notably the lack of standardized scoring systems for evaluating immunoreactivity. The choice of scoring method, in particular the selection of cut-off scores for positivity is rarely addressed. The majority of studies assess positive or negative protein expression based on a pre-determined and often arbitrarily set cut-off score, frequently 10% [24, 25, 29, 30]. Additionally, despite concerns regarding its subjective nature and reproducibility, staining intensity is often incorporated into a variety of scoring systems [31, 32].

While focusing on a single potential predictive marker may provide important information on the association of the protein with tumour response, a multi-marker approach could result in greater sensitivity (and specificity) for the outcome thereby producing more clinically meaningful results. The differential gene and protein expression profiles of rectal cancers following irradiation underline the heterogeneity of this disease which should be reflected in the predictive models used to assess tumour response [33, 34].

The aim of this study was to develop a predictive model of 1) complete pathologic response and 2) complete or partial tumour response to an institutional study using pre-operative HDREB [35, 36] by evaluating the immunohistochemical expression of p53, Bcl-2, vascular endothelial growth factor (VEGF), apoptosis activating growth factor-1 (APAF-1) and epidermal growth factor receptor (EGFR) on 104 pre-treatment rectal biopsies from patients with predominantly cT3 rectal cancer. ROC curve analysis was used to

select relevant cut-off scores for positivity in order to maximize the utility of the immunohistochemistry (IHC) findings.

5.3 Materials and Methods

Pre-operative HDREB

This study was approved by the Research Ethics Committee of the McGill University Health Center. 104 patients with newly diagnosed invasive, resectable rectal adenocarcinoma were included in this study and informed written consent was obtained. Pre-operative staging was performed according to the International Union against Cancer classification and carried out by endorectal ultrasonography and Magnetic Resonant Imaging (MRI). Eligible patients included those with large T2, T3 and early T4 tumours. Patients with abdominal nodal disease, metastases and small T2 tumours with favorable features were excluded from the study. Radiation was delivered pre-operatively with a multi-channel endorectal applicator (Novi Sad and recently with the Oncosmart Nucleotron B.V., Veenendaal, Netherlands) and a high-dose rate remote after-loading system using an Iridium-192 source [37, 38]. A daily fraction of 6.5 Gy was administered over 4 consecutive days to a total of 26 Gy. Each patient was planned with endorectal applicator in place using a CT simulator (Pickler International, Inc, Highland Heights, OH) in order to obtain optimal conformal dosimetry. The dose was prescribed to a clinical target volume that included the gross tumour volume and any intramesorectal deposits visible at MRI. Patients underwent surgery four-to eight weeks after brachytherapy as planned prior to treatment regardless of tumour response.

The assessment of tumour response was performed by pathologic evaluation of rectal specimens post-operatively. Tumours considered to be completely responsive to pre-operative HDREB had no histologic evidence of residual viable carcinoma (ypT0). Partial response was characterized by the presence of micro-foci or foci of residual carcinoma typically ranging from 0.3 cm to 0.9 cm in diameter. Non-responsive tumours were found to have large areas of residual carcinoma ranging in size from 2 cm to 6 cm.

Immunohistochemistry (IHC)

IHC for p53, Bcl-2, VEGF, APAF-1 and EGFR was carried out on formalin-fixed, paraffin-embedded serial sections cut at 3 μm and dried at 37°C overnight. IHC was performed using the avidin-biotin complex (ABC) procedure, including heat-induced epitope retrieval and enzymatic antigen retrieval procedures. Incubation was carried out overnight at 4° C for Bcl-2 (DAKO, clone 124, Denmark, 1:100) and VEGF (Santa Cruz Biotechnology, VEGF-A20, USA, 1:100) and in a moist chamber at 37 ° C for 1 hour for p53 (DAKO, clone DO-7, Denmark, 1:100) and APAF-1 (Novocastra, NCL-APAF-1, 1:100). IHC for EGFR (clone 3C6, 3mg/ml, Ventana Medical Systems, Tucson, USA) was performed using an autostainer according to manufacturer's instructions. Negative controls were treated identically with primary antibodies omitted. Positive controls consisted of colon cancer know to possess mutation of the p53 gene, B-cell lymphoma (Bcl-2), glioblastoma (VEGF), skin (APAF-1) and oral mucosa (EGFR).

Evaluation of IHC

Immunoreactivity was evaluated in a semi-quantitative manner. The proportion of immunoreactive tumour cells over the total number of tumour cells by 5% increments (0%, 5%, 10%, ..., 100%) was determined by three pathologists (A.L., J.J., S.H.) for EGFR and by four pathologists for p53, Bcl-2, VEGF and APAF-1 (C.C.C., A.L., J.J., R.P.M). Only areas of invasive carcinoma were analyzed. Staining was assessed in the nucleus for p53, in the cytoplasm for VEGF, Bcl-2 and APAF-1 and in both cytoplasm and membrane for EGFR carcinoma. Staining intensity was not evaluated.

Statistical analysis

Inter-observer agreement

The reproducibility of the semi-quantitative scoring method was analyzed using the intra-class correlation coefficient (ICC). The ICC is defined as the ratio of the between-subject variance over the (between-subject + within subject variances) and has previously been used to assess agreement of IHC scores [39].

Selection of cutoff scores for protein positivity

Relevant cut-off scores for tumour positivity were obtained from ROC curve analysis [40]. At each IHC score, the sensitivity and specificity for discrimination of response versus no response was plotted thus generating a ROC curve. The score located closest to the point with both maximum sensitivity and specificity, i.e., the point (0.0, 1.0) on the curve was selected as the cut-off score leading to the greatest number of tumours correctly classified as responsive or non-responsive to therapy.

Reproducibility of ROC curve analysis

In order to determine whether ROC curve analysis was a reproducible method for selecting the cut-off scores for p53, Bcl-2, VEGF, APAF-1 and EGFR, ROC curves were generated for each independent pathologist. In addition, 1000-bootstrapped replications were performed to re-sample the data and determine the reliability of the cut-off scores obtained by each scorer. With bootstrapping, 1000 re-samples of equal size were created and ROC curve analysis was performed for each re-sample. Finally, the scores for each tumour were averaged. The final ROC curve resulting from the average scores was used to select the cut-off scores used to predict complete response and also complete or partial response to pre-operative HDREB. The most frequently obtained cut-off score (mode), the mean (95%CI) score over the 100 re-samples and the area under the ROC curve (AUC) and 95%CI were acquired for each analysis. AUCs summarize the discriminatory power for tumour response of each protein over the entire range of scores with values of 0.5 indicating low power and those closer to 1.0 higher power.

Association with clinico-pathological features at the respective cut-offs

The association of tumour response with both clinico-pathological features and protein expression classified as positive or negative around their respective cut-off scores was analyzed with logistic regression. The p-values, odds ratio (OR) and 95%CI for each analysis were obtained. All variables significant (p-value <0.05) in univariate analysis were entered into a multivariate logistic regression model. A selection procedure was used to identify the independent predictors of tumour response. The reliability of the model was established by 1000-bootstrapped replications of the data. The most frequently

selected model from the 1000 sub-samples was chosen as the final predictive model. The sensitivity and specificity of the final models predicting complete response and also complete or partial response was carried out using 100-fold cross-validation.

5.4 Results

Patients

Thirty-two patients were non-responsive to treatment (30.8%), 33 (31.7%) had a complete pathologic tumour response while 39 (37.5%) were found to have partial tumour regression. Tumours lacking sufficient invasive tumour for immunohistochemical evaluation were excluded from the study.

Inter-observer agreement

The inter-observer agreement for p53, Bcl-2, VEGF and APAF-1 is reported elsewhere [41]. The ICC for EGFR was 0.71 (0.68-0.73) indicating strong inter-observer reliability.

Selection of cut-off scores

ROC curve analysis was performed using the average IHC scores. A cut-off score of 50% for p53, 21% for VEGF, 20% for Bcl-2 and EGFR and 10% for APAF-1 was obtained for the analysis of complete tumour response (Figure 1) while cut-off scores of 40% for p53, 53% for VEGF, 1.25% for Bcl-2, 2.5% for APAF-1 and 18% for EGFR (Figure 2) were produced when evaluating complete or partial tumour response. Tumours with scores above the obtained cut-off values were considered positive for the expression of the protein.

Univariate analysis

Complete response versus partial or no response (Table 1)

An association was found between age (p-value = 0.025) and complete response. Patients with moderately or poorly differentiated tumours were predominantly partially or non-responsive to treatment (p-value <0.001). Negative VEGF expression (p-value = 0.004) and EGFR positivity (p-value = 0.003) were significantly associated with complete tumour response.

Complete or partial response versus no response (Table 2)

Moderately or poorly differentiated tumours were more frequently non-responsive to therapy (p-value = 0.042). APAF-1 (p-value = 0.015) and EGFR positivity (p-value = 0.027) were significantly associated with tumour response.

Multivariate logistic regression models

Only VEGF (p-value = 0.003) and EGFR (p-value = 0.006) were selected as independent predictors of complete pathologic response (Table 3). VEGF negative/ EGFR positive tumours had the highest likelihood of completely responding to therapy. Contrarily, VEGF positive/ EGFR negative tumours were least likely to respond with a probability of 6%. Tumours with VEGF positivity/ EGFR positivity or VEGF negativity/ EGFR negativity had a similar probability of response of 30%. The cross-validated sensitivity and specificity of the model were 94% and 45% respectively.

In a multivariate analysis of complete or partial response, only EGFR was selected as an independent predictive factor (p-value = 0.027; OR (95%CI) = 3.11 (1.1-8.5)). Tumours positive for EGFR had a 78% chance of response whereas those negative for the protein had a probability of 54%. The cross-validated sensitivity and specificity of EGFR for complete or partial response to therapy were 58% and 69% respectively.

5.5 Discussion

Pre-operative radiotherapy for patients with locally advanced rectal cancer is based on the premise that irradiation can result in tumour regression thus leading to improved local control, survival and possibly an increased frequency of sphincter-sparing procedures or a more conservative treatment approach [6-8, 13, 14, 42]. Several potential tumour markers governing tumour cell proliferation, cell-cycle arrest or apoptosis have been extensively studied by IHC but often yield contradictory results [20-26, 30]. Despite the fact that IHC is clearly an indispensable research tool, it is recognized that the lack of standardized scoring systems, the inconsistent and often arbitrary selection of cut-off scores for defining tumour "positivity" and the subjective assessment of staining intensity limit the clinical utility of immunohistochemical findings [27, 31].

We have previously shown that a scoring system based on the percentage of positive tumour cells leads to a more complete assessment of the prognostic value of several tumour markers assessed by IHC in colorectal cancer over methods using a pre-determined cut-off score to categorize positive or negative expression [43-46]. We have also demonstrated that this scoring system is reproducible among pathologists in rectal tumour

biopsies for p53, Bcl-2, VEGF and APAF-1 as well as in tissue microarray punches from over 1000 colorectal cancers for EGFR and APAF-1 (ICC = 0.75 and 0.86 respectively, unpublished data) [41]. We again validate these results in this study on rectal tumour biopsies by demonstrating strong inter-observer reliability of EGFR scores between 3 pathologists (ICC = 0.71).

One of the advantages of quantifying IHC scores at the outset is that more relevant cut-off scores for characterizing "positive" protein expression can be established. ROC curves are commonly used in clinical oncology to determine the threshold value above which a test result should be considered positive for some outcome[47-49]. We applied the same principle in this study to determine the cut-off scores above which p53, Bcl-2, VEGF, APAF-1 and EGFR should be considered positive with complete tumour response followed by complete or partial response as the endpoints. The reproducibility of this method was validated by generating ROC curves for each pathologist's scores in addition to re-sampling of the data.

The results of this study show that the combined analysis of VEGF and EGFR expression is highly predictive of complete pathologic response. Tumours considered negative for VEGF and positive for EGFR according to their respective cut-off scores were most likely to be completely responsive to treatment while those positive for VEGF and negative for EGFR (approximately 15% of tumours) were responsive in only 6% of these cases. The sensitivity of the combined markers was 94%. This result indicates that in order to maximize the treatment of potentially completely responsive tumours all patients

could be treated with the exception of those exhibiting VEGF positivity and simultaneous EGFR negativity as their probability of complete response is low.

The expression of EGFR was significantly associated with complete or partial response to therapy. EGFR positive tumours were more than 3 times more likely to respond to treatment compared to EGFR negative tumours. However the sensitivity and specificity of EGFR for tumour response was 58% and 69% respectively. Since complete or partial tumour response is found in approximately 2/3 of patients at the outset, the value of EGFR to predict response is questionable.

The predictive value of EGFR as a marker of response to conventionally fractionated pre-operative radiotherapy in patients with locally advanced rectal carcinoma has been investigated by Giralt *et al.* who described a response rate of 62% in patients with EGFR negative tumours of which 38% had complete pathologic response [50]. In a larger study of 85 patients by the same group positive EGFR expression was associated with lack of complete tumour regression [51]. A high level of EGFR was found to significantly predict decreased tumour downstaging after pre-operative chemo-radiotherapy in a study on 183 patients [52].

Although our results appear to contradict these findings there is evidence to suggest that the predictive value of EGFR positivity for tumour response may be dependent on the dose fractionation regimens. A large randomized controlled trial in head and neck squamous cell carcinoma (HNSCC) (the CHART Head and Neck Trial) investigated the

effect of conventional fractionation (total dose of 66 Gy in 2-Gy fractions over 45 days) or continuous hyper-fractionated accelerated radiotherapy (CHART) (1.5 Gy per fraction, 3 times a day over 12 consecutive days) on pre-treatment EGFR expression in tumour biopsies and overall 3-year loco-regional tumour control [53]. Patients with high EGFR expression receiving CHART had a significantly greater loco-regional tumour control than those undergoing conventional fractionation. Patients with low pre-treatment EGFR expression regardless of the treatment arm had similar probabilities of tumour control. Positive EGFR expression was predictive of a benefit from accelerated radiotherapy relative to conventional fractionation.

Eriksen *et al.* analyzed pre-treatment biopsies from 336 patients participating in the Danish Head and Neck Cancer group designed to evaluate EGFR expression and local tumour control at 5.5 and 6.6 weeks with accelerated radiotherapy and at 9.5 weeks with split-course radiotherapy [54]. Again, a larger benefit from accelerated fractionation was reported in the EGFR-high group.

VEGF is an important mediator of tumour angiogenesis [55]. Its expression is absent in normal colon tissue but is up-regulated in adenoma and significantly over-expressed in carcinomas [56]. VEGF expression has been correlated with tumour aggressiveness, poor survival and liver metastases [29, 57-60]. Regulation of VEGF is influenced by cytokines, inactivation of tumour suppressor genes such as p53 and oncogenic activation including KRAS mutation [58, 61, 62]. VEGF has been linked to Bcl-2 expression in colorectal cancer and acts as a survival factor [63]. Moreover, VEGF mRNA is significantly up-regulated in

response to hypoxia which can occur in growing tumours whose oxygen and nutrient requirements surpass the diffusional capacity of the host vasculature [64]. It is known that hypoxic tumours are more radio-resistant than their normoxic counterparts. An increased expression of VEGF may be reflective of the hypoxic state of the tumour. This observation is in line with the results of this study which demonstrate that completely responsive tumours are most frequently negative for the protein compared to partially or non-responsive tumours.

We acknowledge that pre-operative HDREB remains an experimental approach. However the long-term data are very favorable and this novel modality is presently being considered for a randomized trial. At the present time, different radiation schedules are used: in northern Europe, 25 Gy in 5 fractions (short course) is commonly applied, whereas 45 Gy in 25 fractions (long course) with chemotherapy is preferred in southern Europe and North America. Bujko *et al.* randomized 310 patients with cT3 rectal cancer to 5 Gy x5 followed by surgery or conventional preoperative 50.4 Gy plus bolus 5FU1leucovorin daily over 5 weeks followed by surgery and reported similar local control and survival results [10]. The ability to predict complete pathologic response or sensitivity to radiation based on IHC would have a significant impact on the selection of patients for pre-operative radiotherapy or chemo-radiation therapy schedule.

5.6 Conclusion

In conclusion, the combined analysis of VEGF and EGFR is predictive of complete pathologic tumour response to pre-operative HDREB. The predictive value of EGFR may be dependent on dose-fractionation. A large-scale prospective study is necessary to validate these preliminary findings.

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Table 1: Patient characteristics and their association with tumour response to pre-operative brachytherapy. CR = complete response, PR = partial response, NR = no response, N = number.

		CR	PR + NR	P-value	OR (95%CI)
		N (%)	N (%)		
Sex	Male	23 (32.9)	47 (67.1)	0.7234	0.85 (0.35-2.07)
	Female	10 (29.4)	24 (70.6)		
Age (mean ± SD)		69.3 ± 10.9	63.0 ± 11.3	0.025	1.05 (1.0-1.09)
Tumour grade	Well	12 (50.0)	6 (11.3)		
	Moderate	12 (50.0)	43 (81.1)	<0.001	0.13 (0.04-0.41)
	Poor	0 (0.0)	4 (7.6)		
VEGF	≤ 21%	14 (56.0)	14 (23.0)	0.004	0.23 (0.09-0.63)
	>21%	11 (44.0)	47 (77.0)		
EGFR	≤ 20%	6 (27.3)	34 (63.0)	0.003	5.78 (1.85-18.07)
	>20%	16 (72.7)	20 (37.0)		
Bcl-2	≤ 20%	17 (68.0)	43 (81.1)	0.203	2.02 (0.68-5.99)
	>20%	8 (32.0)	10 (18.9)		
APAF-1	≤ 10%	14 (53.9)	41 (70.7)	0.137	2.07 (0.8-5.38)
	>10%	12 (46.2)	17 (29.3)		
p53	≤ 50%	14 (53.9)	38 (64.4)	0.359	1.55 (0.61-3.96)
	>50%	12 (46.2)	21 (35.6)		

Table 2: Patient characteristics and association with complete or partial tumour response to pre-operative brachytherapy. CR=complete response, PR=partial response, NR=no response, N = number.

		CR + PR	NR	P-value	OR (95%CI)
		N (%)	N (%)		
Sex	Male	46 (65.7)	24 (34.3)	0.268	1.7 (0.67-4.3)
	Female	26 (76.5)	8 (23.5)		
Age (mean ± SD)		66.2 ± 11.4	63.8 ± 11.5	0.320	1.02 (0.98-1.06)
Tumour grade	Well	16 (30.2)	2 (8.3)		
	Moderate	35 (66.0)	20 (83.3)	0.042	0.31 (0.1-0.96)
	Poor	2 (3.8)	2 (8.3)		
APAF-1	≤ 2.5%	6 (17.7)	22 (44.0)	0.015	3.67 (1.29-10.41)
	>2.5%	28 (82.4)	28 (56.0)		
EGFR	≤ 18.3%	8 (21.6)	18 (46.2)	0.027	3.11 (1.14-8.48)
	>18.3%	29 (78.4)	21 (53.9)		
p53	≤ 40%	10 (25.6)	17 (37.0)	0.266	1.7 (0.67-4.33)
	> 40%	29 (74.4)	29 (63.0)		
VEGF	≤ 52.5%	16 (43.2)	13 (26.5)	0.107	0.47 (0.19-1.18)
	>52.5%	21 (56.8)	36 (73.5)		
Bcl-2	≤ 1.25%	9 (36.0)	15 (28.3)	0.493	0.7 (0.26-1.93)
	>1.25%	16 (64.0)	38 (71.7)		

Table 3: Characteristics of the logistic regression model of complete tumour response

	Estimate (95%CI)	Standard error	P-value	OR (95%CI)
Intercept	-0.852	0.56	0.128	
VEGF	-1.965	0.67	0.003	0.14 (0.04-0.52)
EGFR	1.904	0.69	0.005	6.71 (1.75-25.73)

Figure legends

Figure 1: ROC curves for complete tumour response generated from the analysis of average protein expression of p53 (A), Bcl-2 (B), VEGF (C), APAF-1 (D) and EGFR (E). Arrows indicate the point on the curve corresponding to the selected cut-off score. AUC = area under the curve and 95%CI.

Figure 2: ROC curves for complete or partial tumour response generated from the analysis of average protein expression of p53 (A), Bcl-2 (B), VEGF (C), APAF-1 (D) and EGFR (E). Arrows indicate the point on the curve corresponding to the selected cut-off score. AUC = area under the curve and 95%CI.

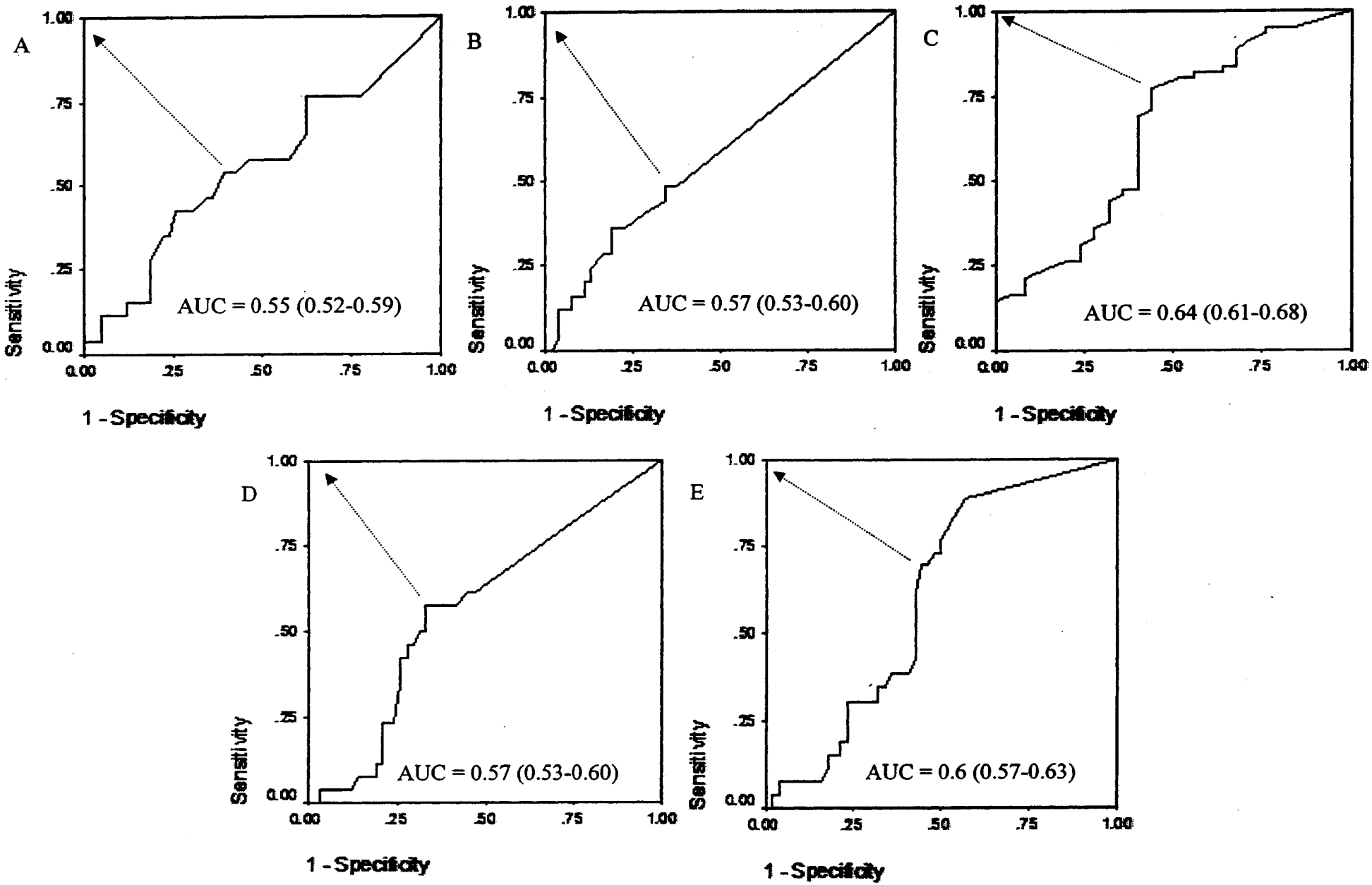
A
AUC = 0.55 (0.52-0.59)
B
AUC = 0.57 (0.53-0.60)
C
AUC = 0.64 (0.61-0.68)
D
AUC = 0.57 (0.53-0.60)
E
AUC = 0.6 (0.57-0.63)
Sensitivity
1 - Specificity
0.00
.25
.50
.75
1.00

Figure 1

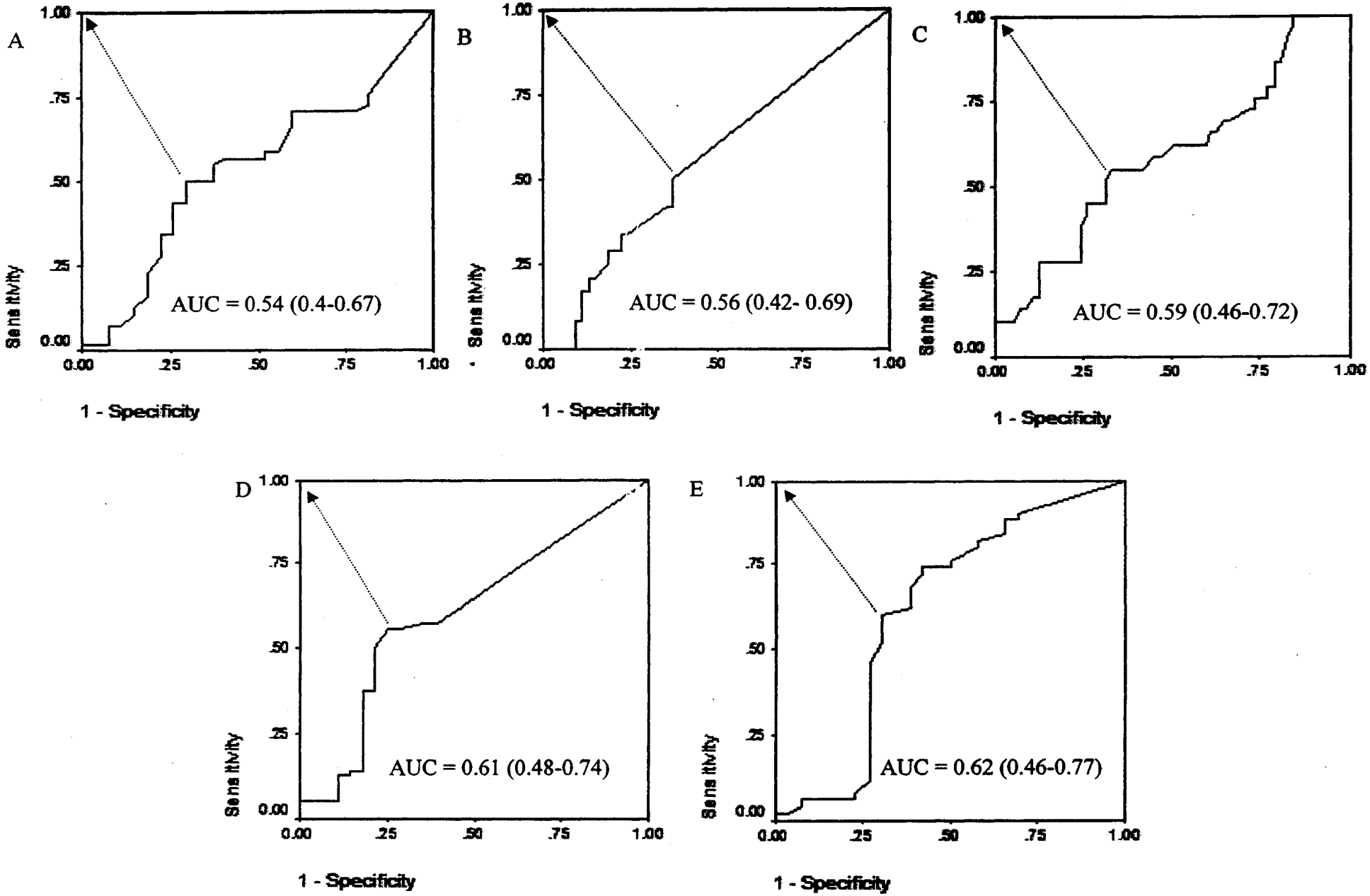
A
B
C
D
E
Sensitivity
1 - Specificity
0.00
.25
.50
.75
1.00
AUC = 0.54 (0.4-0.67)
AUC = 0.56 (0.42- 0.69)
AUC = 0.59 (0.46-0.72)
AUC = 0.61 (0.48-0.74)
AUC = 0.62 (0.46-0.77)

Figure 2

CHAPTER 6: Summary and Conclusion

6.1 Discussion

Tumour regression following pre-operative radiotherapy is associated with improved tumour control and survival rates in patients with locally advanced rectal cancer [1-13]. The ability to predict which patients could have complete pathologic response or tumour regression prior to treatment would have a significant impact on the selection of patients for pre-operative radiotherapy and could potentially modify post-treatment surgical and clinical planning.

IHC is commonly carried out on pre-treatment biopsy specimens from patients undergoing pre-operative radiotherapy in order to identify tumour markers which may have predictive value [14-23]. Despite the fact that IHC is clearly an indispensable research tool, it is recognized that the lack of standardized scoring systems, the inconsistent and often arbitrary selection of cut-off scores for defining tumour "positivity" and the subjective assessment of staining intensity limit the clinical utility of immunohistochemical findings [19, 23-28]. These factors undoubtedly contribute to the contradictory results often found between similar studies on the same protein.

Colorectal cancer is a heterogeneous disease, a fact underlined by the differential gene and protein expression profiles of tumours following radiotherapy [29, 30]. Therefore, rather than focusing on a single potential predictive marker of tumour response, it would be more beneficial to use a multi-marker approach resulting in greater sensitivity and specificity for tumour response thereby improving the clinical significance of results.

The present study was undertaken to identify relevant tumour markers of response to pre-operative HDREB and to develop a multi-marker model which would allow one to predict the probability of complete tumour response and complete or partial response for each combination of selected markers. The proteins in this study were chosen for their roles as mediators of tumour response to ionizing radiation and included p53, Bcl-2, VEGF, APAF-1, and EGFR [22, 31-43]. The expression of these proteins was evaluated using IHC on pre-treatment biopsy specimens from 104 patients undergoing pre-operative HDREB [44, 45]. In addition to the combined analysis of these proteins, a novel method for selecting relevant cut-off scores to define tumour "positivity" for each marker, namely ROC curve analysis was used in conjunction with a quantitative scoring method for assessing immunoreactivity [46].

6.1.1 Preliminary analyses

The preliminary findings outlined in Chapter 2 were obtained by analyzing IHC scores from one pathologist who evaluated the expression of p53, Bcl-2, VEGF and APAF-1. It was established that the absence of VEGF was strongly predictive of complete pathologic response to pre-operative HDREB. Patients with complete response were predominantly those with tumours expressing VEGF in less than 20% of cells whereas non-responsive tumours were largely immunoreactive in more than 80% of cells. Regardless of the scoring system used to analyze the distribution of VEGF scores, the results were consistent. Moreover, multivariate analysis of complete or partial tumour response using CART selected VEGF as the most important predictive factor in patients undergoing pre-operative HDREB. The expression of APAF-1 was also investigated in the pre-treatment

biopsies of these patients and found to be significantly associated with improved response to treatment. Ionizing radiation is a known stimulus of apoptosis [47, 48]. Irradiation leads to disruption of the mitochondrial membrane and release of cytochrome c which is free to bind APAF-1, the core protein forming the apoptotic machinery known as the apoptosome [34, 49]. Activation of APAF-1 is followed by recruitment of caspases leading to a proteolytic cascade resulting in cell death [34, 49]. The extent of apoptosis following pre-operative radiotherapy has been shown to play an important role in tumour regression [50]. The results of this study are in line with these findings. Higher APAF-1 protein expression levels may be reflective of a greater susceptibility of the tumour cells to undergo apoptosis and therefore respond to radiotherapy.

CART analysis was included in this Chapter to identify associations of the proteins with tumour response. It was only possible to employ this statistical approach because the immunoreactivity for each protein was assessed semi-quantitatively on a scale from 0% to 100%. The findings in Chapter 2 were of utmost importance. They led to the fundamental realization that the full potential of IHC was limited to the choice of scoring method and the selection of cut-off scores for defining tumour "positivity".

6.1.2 Semi-quantitative assessment of immunoreactivity

The majority of studies evaluating IHC in colorectal cancer assess positive or negative protein expression based on a pre-determined and often arbitrarily set cut-off score, frequently 10% [19, 23, 25, 27]. More detailed analysis of protein expression over a larger range of values could contribute substantially to the predictive or prognostic value of

tumour markers. By evaluating the proportion of immunoreactive tumour cells over the total number of tumour cells, IHC scores can be assigned percentages ranging from 0% to 100%. One of the advantages of semi-quantitative scores is that more powerful statistical techniques can be applied to identify relationships between proteins and their outcomes [51, 52]. Most importantly, by evaluating scores semi-quantitatively at the outset, more clinically relevant cut-off scores for tumour positivity can be selected.

Prior to its implementation however, the inter-observer reproducibility of this scoring method needed to be determined. In Chapter 3, the inter-observer agreement of IHC scores between four pathologists for the proteins p53, Bcl-2, APAF-1 and VEGF was assessed. Semi-quantitative scoring was found to be reproducible for all markers with the greatest agreement occurring for the proteins p53 and VEGF. Inter-observer reproducibility was decreased for Bcl-2 and APAF-1 and could be explained mainly by weak staining of tumour cells. The average scores for each pathologist and the overall mean protein expression were determined. Pathologist 1 consistently "over-scored" compared to Pathologist 2 and 4 while Pathologist 3 "under-scored" for each protein. These results underlined the importance of obtaining scores from multiple observers and demonstrated that in order to compensate for over- or under-scoring, the average protein expression for all observers would be a more accurate estimate of the extent of immunoreactivity in the tissue.

6.1.3 ROC curve analysis

ROC curves were developed in the 1950s and used for signal detection experiments involving radars [46]. However, their application to medical science has soared over the last 15 years and they are currently used to determine the discriminatory power of quantitative diagnostic indices and to compare the performance of several different tests to discriminate between patients with or without an outcome of interest [53-61]. ROC curves can also be used to determine an appropriate threshold value above which a test result should be considered positive for an outcome. For example, it is recommended that patients with prostate-specific antigen (PSA) levels of 4.0ng/mL or greater undergo prostatic biopsy. This threshold value was obtained by ROC curve analysis [58].

The same principle of threshold values could be applied to the selection of cut-off scores for determining tumour positivity following IHC. In Chapter 4, cut-off scores for the protein RHAMM were investigated using a tissue microarray of 1197 colorectal cancers with complete clinico-pathological data [62]. RHAMM expression was scored in each tissue microarray punch using the semi-quantitative scoring method and ROC curve analysis was performed. The cut-off score was chosen such that the trade-off between sensitivity and specificity was the smallest, i.e., the cut-off score maximized the number of correctly classified tumours with and without the clinico-pathological feature. For T stage, N stage, tumour grade and vascular invasion, the cut-off score was found to be 100% (<100% versus 100% immunoreactive tumour cells) and for survival, 90% (<90% versus ≥90% immunoreactive tumour cells). In order to validate these findings, re-sampling of the data by 100 bootstrapped replications was performed. ROC curve analysis was carried out for

each of the 100 re-samples and the cut-off score was obtained every time. The cut-off value was 100% for T stage, N stage, tumour grade, vascular invasion and 90% for survival thereby confirming the previous results.

The findings of this study indicated that ROC curve analysis was a viable alternative to the selection of IHC cut-off scores for tumour positivity. Moreover, the cut-off scores obtained for the features under investigation were highly reproducible.

6.1.4 Applying ROC Curves: Some Considerations

Several considerations should be taken into account regarding the use of ROC curves to select cut-off scores for positivity, particularly for novel tumour markers with no previously established cut-off score. IHC should ideally be performed on a large number of tumours. The greater the sample size, the more accurate the cut-offs obtained from ROC curve analysis. Smaller sample sizes will inevitably result in more variable cut-off scores. However, re-sampling of the data by bootstrapping may compensate to some extent for small sample sizes.

ROC curve analysis is carried out on features with binary outcomes. In this study, T stages were combined into early (T1+T2) and late (T3+T4) stages. This dichotomy may not however be suitable for different tumour types.

It should also be emphasized that categorizing protein expression around the selected cut-off score does not imply that significant statistical associations with the outcome will

occur. However, significant associations may be more biologically meaningful and more likely to occur when appropriate cut-off scores are used to assess positivity.

Finally, the cut-off scores were selected in this study such that the trade-off between sensitivity and specificity was the smallest, therefore leading to the greatest overall number of correctly classified tumours with and without the clinico-pathological feature. However, it may be more beneficial when investigating other outcomes, such as response to treatment to choose a cut-off score leading to higher sensitivity over specificity. This would increase the probability of selecting those patients most likely to be responsive to treatment.

6.1.5 Reproducibility of cut-off scores between pathologists

Having demonstrated the application of ROC curve analysis using scores from one pathologist on the novel tumour marker RHAMM [62], the next objective was to integrate the findings from Chapters 3 and 4 into a cohesive study that illustrated the full potential of ROC curves in conjunction with the semi-quantitative scoring method to identify associations with a well-known tumour marker and clinico-pathological outcomes. More specifically, this involved establishing whether the semi-quantitative scoring method found to be reproducible in biopsy specimens was also reliable in a large number of tumours using a tissue microarray, determining to what extent cut-off scores selected by ROC curve analysis varied between different pathologists, identifying the cut-off values using the average scores and finally categorizing the data around these cut-offs to

determine the association of the tumour marker with T stage, N stage, tumour grade, vascular invasion and survival.

For this undertaking, the protein EGFR was chosen for several reasons. First, the prognostic and predictive value of EGFR varies significantly in the literature [41, 63-69]. Second, several scoring methods have been proposed for the assessment of EGFR immunoreactivity [70-72], and third, there is considerable interest in EGFR as a target for therapeutic intervention [73].

The proposed semi-quantitative scoring method resulted in strong inter-observer agreement between pathologists (ICC = 0.86), validating the findings in Chapter 3 and further supporting the averaging of scores to obtain a more accurate estimate of the "true" EGFR immunoreactivity for each tumour punch. Moreover, the selected cut-off scores for each pathologist were highly consistent across the clinico-pathological features, further confirming the reproducibility of ROC curve analysis. Finally, an important association of EGFR with survival time was identified both in univariate and multivariate analysis, indicating that EGFR could be used as a prognostic marker in patients with colorectal cancer.

6.1.6 Predictive model of tumour response

The final objective of the research project involved the development of a predictive model of tumour response to pre-operative HDREB which could be established more

accurately by incorporating the tools introduced in Chapters 3 and 4 for assessing immunoreactivity.

The reproducibility of the quantitative scoring method although confirmed for EGFR on the tissue microarray, required additional validation in the rectal biopsy specimens from the patients undergoing HDREB. EGFR in biopsies was scored by three pathologists. The ICC was determined to be 0.71 indicating strong inter-observer agreement. ROC curve analysis was performed on the average scores for all 5 proteins namely p53, Bcl-2, VEGF, APAF-1 and EGFR using first complete response followed by complete or partial tumour response as endpoints. Appropriate cut-off scores for positivity were obtained in each case. Information with respect to age, sex and tumour grade was available for most patients.

Only two features included in the analysis were determined to be independent predictors of complete pathological response: VEGF and EGFR. Tumours with less than an average of 21% staining (negative) for VEGF and more than 20% staining (positive) for EGFR had greater than a 74% chance of responding to treatment. Those with VEGF positive and EGFR negative tumours were least likely to have a complete pathologic response. The odds ratios for the final model illustrate the impact of each predictor for complete response. Patients negative for VEGF were found to respond 7.1 times more than their VEGF-positive counterparts regardless of EGFR expression. Tumours positive for EGFR had a 6.7 times greater chance of complete response compared to EGFR negative tumours adjusting for the effect of VEGF. The combined analysis of VEGF and EGFR

resulted in high sensitivity (94%) and moderate specificity for the outcome (45%). Twenty five patients were simultaneously positive for VEGF and negative for EGFR. Of these, only 1 had a complete response to pre-operative HDREB. Based on these results, it was established that pre-operative HDREB may not be suitable for patients with VEGF-positive, EGFR-negative tumours, approximately 25% of candidates.

Only EGFR was found to have significant predictive value as a marker of complete or partial tumour response to pre-operative HDREB. However, the cross-validated sensitivity (58%) and specificity (69%) for the EGFR model were not encouraging. Since complete or partial response is found in approximately 2/3 (66%) of patients undergoing this treatment at the outset, the importance of this finding is negligible at this time.

6.1.7 VEGF and EGFR

VEGF is a mitogen, a survival factor, a potent mediator of angiogenesis and a facilitator of metastatic spread [74-77]. Its expression has been linked to poor clinical outcome, tumour aggressiveness and as demonstrated in this study, lack of response to pre-operative radiotherapy [78-80]. VEGF is up-regulated by inactivation of p53, oncogenic activation of Bcl-2 and by hypoxia [25, 39, 79, 81-85]. Tumour cells living in a hypoxic environment require nutrients and oxygen that can no longer be supplied by the host vasculature[76, 79, 86]. These hypoxia-resistant cells express VEGF which, when bound to receptors on endothelial cells, leads to up-regulation of proteins involved in degradation of the surrounding matrix, endothelial cell proliferation, migration and formation of new vessels capable of increasing the demands made by starving tumour cells. It is known that hypoxic cells are

considerably resistant to irradiation [74, 75]. Indeed the presence of oxygen is a fundamental requirement for radiation to kill cells [86]. It can therefore be hypothesized that up-regulated VEGF expression occurs in more hypoxic-resistant tumours which sustain irradiation on the basis that they are oxygen-deprived. The greater the extent of VEGF expression, the more resistant the tumour cells to pre-operative radiotherapy. This hypothesis is further validated by the fact that the cut-off scores used to discriminate between complete responders and partial or non-responders were considerably lower (21%) than the cut-off score required to classify complete or partial responders (<50% staining) versus non-responders. A similar VEGF gradient was found in Chapter 2 using CART analysis.

Therapies targeting VEGF and its signaling pathway are currently being studied in patients with several tumour types including colorectal cancer [87]. There is evidence to suggest that inhibition of VEGF reduces angiogenesis in tumours and interferes with tumour growth and metastasis [87]. VEGF is known to increase vascular permeability which leads to a reduction in the efficient delivery of chemotherapeutic agents to the tumour [88]. The monoclonal antibody Bevacizumab (Avastin©) has been approved for use in combination with FU-based chemotherapy for the first-line treatment of patients with metastatic colorectal cancer [89]. Anti-VEGFR therapy in combination with pre-operative radiotherapy could substantially increase tumour regression especially in patients undergoing a HDREB protocol.

EGFR is activated following both single and repeated doses of ionizing radiation [90]. Schmidt-Ullrich *et al* found that daily fractions of 2 Gy over the course of 30 days led to a nine-fold increase in EGFR mRNA levels *in vitro* [90]. Several studies in colorectal cancer have shown that over-expression of EGFR in pre-treatment tumour biopsies from patients undergoing pre-operative radio- or radiochemotherapy was associated with a lack of tumour response [37, 91, 92]. The findings of the current study appear to contradict these reports. Over-expression of EGFR was significantly associated with tumour response to pre-operative HDREB in both univariate and multivariate analysis.

These results can be explained by examining the dose fractionation regimens administered to patients undergoing radiotherapy. A large randomized controlled trial in head and neck squamous cell carcinoma (HNSCC) (the CHART Head and Neck Trial) investigated the effect of conventional fractionation or continuous hyper-fractionated accelerated radiotherapy (CHART) (1.5 Gy per fraction, 3 times a day over 12 consecutive days) on pre-treatment EGFR expression in tumour biopsies and overall 3-year loco-regional tumour control [93]. Patients with high EGFR expression receiving CHART had a significantly greater loco-regional tumour control than those undergoing conventional fractionation. Patients with low pre-treatment EGFR expression regardless of the treatment arm had similar probabilities of tumour control. Positive EGFR expression was predictive of a benefit from accelerated radiotherapy relative to conventional fractionation. Eriksen *et al.* [94] analyzed pre-treatment biopsies from 336 patients participating in the Danish Head and Neck Cancer group designed to evaluate EGFR expression and local tumour control at 5.5 and 6.6 weeks with accelerated

radiotherapy and at 9.5 weeks with split-course radiotherapy. Again, a larger benefit from accelerated fractionation was reported in the EGFR-high group.

The predictive value of EGFR therefore appears to be dependent on the dose of radiation and the time course of treatment. The expression of EGFR is hypothesized to play a crucial role in tumour cell repopulation following irradiation by activating MAP kinase and PI3-kinase signaling pathways [90]. Though the aim of ionizing radiation is to kill cells, it has been shown *in vitro* that repeated exposures of radiation between 1.0 and 2.0 Gy actually stimulate EGFR and its downstream effectors in cells which have survived irradiation [90]. Conventional external beam radiation is given in 1.8 Gy fractions, 25 times to a total of 45 Gy. In the CHART trial however, 1.5 Gy were administered 3 times a day (4.5 Gy daily) for 12 days [93]. Pre-operative HDREB is based on 6.5 Gy daily for 5 days to a total of 25 Gy [44]. Both these regimens significantly shortened treatment time and used high doses of radiation daily which could prevent repopulation of tumour cells leading to local failure.

In summary, the combined analysis of VEGF and EGFR is predictive of complete pathologic tumour response to pre-operative HDREB. Although EGFR alone was found to independently predict tumour response, the low sensitivity and specificity of the model may have limited value. A large-scale prospective study is necessary to validate these findings for patients undergoing pre-operative HDREB.

6.2 Conclusion

While the finding that the combined analysis of VEGF and EGFR predicts complete pathologic response to pre-operative HDREB may not allow itself to generalization to other forms of pre-operative radiotherapy for rectal cancer, the novel method proposed and validated in this study to evaluate IHC could lead to significant changes in how scoring systems are used to maximally extract useful information from the expression of proteins and correlate them with clinical endpoints. More specifically, the results of this study show that evaluating immunoreactivity in a quantitative manner is reproducible among pathologists and that ROC curve analysis can be applied in conjunction with this scoring method to select more relevant cut-off scores that should increase the clinical utility of IHC findings.

The ROC methodology proposed in this study is included in several papers currently in press which evaluate the expression of proteins MST1, RKIP, TGF-β and members of the TGF-β signaling pathway in colorectal cancer [95-97]. Additionally, this method has been used to determine cut-off scores for 20 potential tumor markers in order to determine their association with tumour budding in colorectal cancer [98].

6.3 Original contributions to science

- First published report on APAF-1 IHC in colorectal cancer as well as on the predictive value of APAF-1 to pre-operative radiotherapy.
- Proposal and validation of a quantitative scoring system for describing tumour immunoreactivity
- Development of a novel method for determining cut-off scores for tumor positivity using a quantitative scoring system (ROC curve analysis)
- First study assessing the predictive value of p53, Bcl-2, VEGF, APAF-1 and EGFR to pre-operative HDREB

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VEGF as a Predictive Marker of Rectal Tumor Response to Preoperative Radiotherapy

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BACKGROUND. Neoadjuvant radiotherapy for rectal cancer may result in tumor downstaging or complete tumor regression leading to greater sphincter preservation. The identification of molecular predictive markers of tumor response to preoperative radiotherapy would provide an additional tool for selecting patients most likely to benefit from treatment. The aim of this study was to determine whether VEGF expression in preirradiation tumor biopsies is a useful predictive marker of tumor response in patients with rectal cancer undergoing preoperative radiotherapy.

METHODS. Immunohistochemistry for VEGF was performed on 59 preirradiation biopsies from patients with completely responsive (ypT0) or nonresponsive tumors after preoperative radiotherapy. VEGF positivity was evaluated using several scoring methods and the association between VEGF and tumor response was compared. The distribution of VEGF scores was obtained as well as the mean VEGF expression in the two response groups.

RESULTS. The mean VEGF expression in nonresponsive tumors (NR) was significantly greater than in completely responsive tumors (CR) ($P = 0.0035$). Nearly half (47%) of all CR tumors had a VEGF expression of 10% or less. Eleven tumors were negative (0% immunoreactivity) for the protein and all of these (100%) were complete responders. Fifty-two percent of the NR tumors had VEGF scores of 80% or greater. The four scoring methods used to determine the association between VEGF and tumor response each produced significant results ($P < 0.05$).

CONCLUSIONS. The results of this study indicate that VEGF assessed immunohistochemically from preirradiation tumor biopsies may be a useful marker of rectal tumor response to preoperative radiotherapy. *Cancer* 2005;104:2517–21.

KEYWORDS: rectal cancer, VEGF, predictive marker, preoperative radiotherapy.

Neoadjuvant radiotherapy is part of standard care for patients with advanced rectal cancer.[1] This treatment has been shown to improve survival and may reduce local recurrence rates versus surgery with or without postoperative radiotherapy.[2] In addition, tumor downstaging and complete tumor regression may be achieved with preoperative radiotherapy leading to greater sphincter preservation.[3,4] The ability to predict tumor response from preirradiation biopsies may significantly improve the selection of patients for preoperative radiotherapy.

Vascular endothelial growth factor (VEGF) is a potent mediator of tumor angiogenesis.[5] VEGF can be activated in tumor cells by several factors, including oncogenes, tumor suppressor genes, cytokines (IL-1, IL-6), and hypoxia resulting in secretion of proteolytic enzymes and matrix metalloproteases that degrade the basement membrane and extracellular matrix surrounding the tumor.[6,7] These events ulti-

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mately lead to endothelial cell migration and the formation of a new vasculature that supports the growth of the tumor and its nutrient requirement.[8] In situ hybridization studies show that VEGF mRNA is significantly elevated in many human cancers and is associated with poor clinical outcome and greater aggressiveness of the tumor.[5,9] VEGF has been shown to up-regulate the antiapoptotic gene BCL-2, thereby acting as a survival factor for both endothelial and tumor cells.[10,11] Activation of VEGF also leads to increased vascular permeability of tumor vessels, causing them to be 'leaky' and less efficient in their ability to diffuse oxygen.[7,12] This leaky vasculature appears to contribute to less efficient delivery of chemotherapeutic agents to the tumor.[10,13]

The aim of this study was to determine, from preirradiation tumor biopsies, the value of VEGF as a predictive marker of rectal tumor response to preoperative radiotherapy.

MATERIALS AND METHODS

Patients

Fifty-nine patients with rectal adenocarcinoma were entered into the study and informed written consent was obtained from each. Clinical staging was performed via magnetic resonance imaging (MRI) and endoscopic ultrasonography (EUS). Patients were treated on a preoperative conformal high-dose rate endorectal brachytherapy protocol.[14] Patients with abdominal nodal disease or metastases were excluded from the study. Radiation was delivered preoperatively with a flexible, 8-channel endorectal catheter using a high-dose-rate remote after-loading system. A daily fraction of 6.5 Gy was administered over 4 consecutive days to a total of 26 Gy. Each patient was planned by computed tomography (CT) simulation before treatment with the endorectal catheter in place. To obtain optimal conformal dosimetry for each individual tumor, differential loading of the eight channels was performed. Patients underwent surgery 4–8 weeks later, regardless of tumor response.

Tumor response was evaluated pathologically from the postoperative specimens. Complete tumor response was defined as no evidence of residual carcinoma or ypT0.[15] Partial response was characterized by the presence of microfoci of residual carcinoma typically measuring from 0.3–0.9 cm in diameter. Nonresponsive tumors have large residual carcinoma with absence of microfoci. Residual tumors ranged from 2–6 cm in diameter. For the purposes of this study, only completely responsive and nonresponsive tumors were evaluated.

Immunohistochemistry

Preirradiation, formalin-fixed paraffin-embedded tumor biopsies from all 59 patients were collected. Immunohistochemistry for VEGF was performed using the avidin-biotin complex (ABC; Vector Laboratories, Burlingame, CA) procedure, including heat-induced antigen retrieval procedures. Incubation with polyclonal anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA; VEGF-A20, 1:100) was carried out at 37 °C for 1 hour. Negative controls were treated identically with primary antibody omitted. Tissue from glioblastoma was used as the positive control.

Scoring of VEGF Immunohistochemistry

Evaluation of VEGF immunoreactivity was made by two independent observers. The percentage of positive tumor cells was determined by each observer and the average of the two scores was obtained.

Several scoring systems have previously been used to evaluate VEGF positivity.[16–18] In this study, the average scores obtained by the observers were used to compare the following scoring methods: 1) negative/positive: negative tumor with 0% VEGF staining versus positive tumor with any degree of staining; 2) 10% cutoff: positive tumor with more than 10% immunoreactive tumor cells; 3) 0, 1+, 2+, 3+: tumor is negative for VEGF (0), has fewer than 20% positive cells (1+), has 20–50% positive staining (2+), or has greater than 50% staining (3+); 4) percentages: the actual percentage of positive tumor cell staining obtained by the observers.

Assessment of VEGF immunoreactivity from preirradiation tumor biopsies was performed blinded to postoperative tumor response.

Statistical Analysis

Patient and tumor characteristics were assessed by the chi-square test. The Wilcoxon Rank Sum test was used to evaluate differences in mean VEGF expression between response groups. $P < 0.05$ was considered statistically significant. Analysis of VEGF immunoreactivity and response was carried out by the Fisher exact and chi-square tests for scoring methods 1–3. Logistic regression was used to test for differences in VEGF and tumor response in scoring method 4. All analyses were carried out using SAS, 8th ed. (SAS Institute, Cary, NC).

RESULTS

Pathologic evaluation of the irradiated tumor bed postoperatively identified 30 tumors with complete response and 29 with no response to radiotherapy.

TABLE 1
Patient and Tumor Characteristics ($N = 59$)

Characteristics	Female	Male
Age in yrs		
Median	65.5	66.4
Maximum	91	88
Minimum	49	38
Tumor stage %		
cT2	5.9	2.9
cT3	94.1	91.2
cT4	0	5.9
Nodal status %		
Positive	35.3	29.4
Negative	64.7	70.6
Tumor response %		
Complete	20.3	30.5
No response	13.6	35.6
Total	33.9	66.1

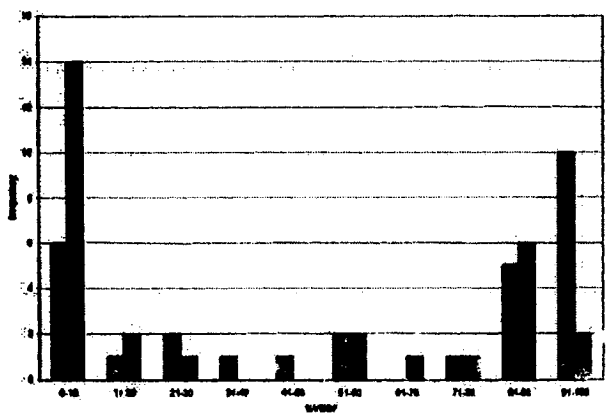

FIGURE 1. Distribution of VEGF scores for the response groups. Complete response: pink bars, no response: blue bars.

Patient and tumor characteristics are summarized in Table 1.

Cytoplasmic immunoreactivity ranged from 0–100%. The mean VEGF expression in nonresponsive (NR) tumors was 63% and was significantly greater than completely responsive (CR) tumors (37.31%) ($P = 0.0035$). No significant association between age, gender, stage, or nodal status and tumor response was found.

The distribution of VEGF scores for each response group is shown in Figure 1. Nearly half (47%) of all CR tumors were found to have a VEGF expression of 10% or less. Of those, 11 tumors (79%) were negative for the protein (no VEGF expression). All NR tumors showed some degree of VEGF positivity. Fifteen of these 29 tumors (52%) had at least 80% immunoreactivity. Ten NR tumors had more than 90% VEGF expression, whereas only 2 CR tumors (6%) were found in this region. The association between VEGF expression and tumor response produced by each of the four scoring systems is listed in Table 2. All methods yielded a statistically significant association between VEGF immunoreactivity and tumor response ($P < 0.05$).

TABLE 2
Comparison of Scoring Methods Used to Determine the Association of VEGF and Tumor Response

Scoring methods	P-values
1) Presence/negative	0.0007[a]
2) 10% cutoff	0.0153[a]
3) 0, 1+, 2+, 3+	0.0026[b]
4) Percentages	0.0172[c]

P-values computed from
[a] Fisher exact test
[b] chi-square test
[c] logistic regression.

These results appear to indicate that tumors completely responsive to preoperative brachytherapy most often express no or low levels of VEGF in their pretreatment biopsies, whereas nonresponsive tumors are generally highly immunoreactive.

DISCUSSION

The identification of molecular predictive markers of tumor response to preoperative radiotherapy would provide an additional tool for selecting patients most likely to benefit from treatment. Recently, the role of VEGF in angiogenesis and, particularly, in colorectal cancer has been investigated. Immunohistochemistry studies have shown VEGF to be absent in normal colorectal mucosa, while carcinomas are highly immunoreactive.[19,20] Wong et al.[20] investigated the temporal relationship between VEGF expression and tumor progression from adenoma to carcinoma. They found that activation of VEGF was an early event in the adenoma-carcinoma sequence, suggesting that VEGF may be an angiogenesis-initiating factor in the early phase of tumor development. In colorectal carcinoma, no difference in stage-specific VEGF expression has yet been reported. Nozue et al.[18] described VEGF status before and after preoperative radiotherapy in locally advanced rectal cancers. They found a greater number of VEGF-positive tumors and more intense VEGF immunoreactivity after treatment. Upregulation of VEGF has been associated with poor prognosis in patients with colorectal cancer and linked to liver metastasis.[21,22]

Hypoxia is a major inducer of VEGF activation, which occurs primarily through the transcription of hypoxia-inducible factor 1-alpha (HIF-1α).[7] Tumor

growth leads to limitations in oxygen diffusion provided by the host vasculature, creating areas of hypoxia.[23] In response to this low oxygen tension, tumor cells either undergo apoptosis or begin to produce VEGF to induce vasculature that will in turn increase oxygen delivery to sustain their survival.[24] In addition, VEGF may activate Bcl-2, an antiapoptotic protein.[10,11] This may further contribute to the survival advantage of tumor cells expressing VEGF.

Our results show that low or absent VEGF in preirradiation rectal tumor biopsies is strongly associated with complete tumor response. A comparison of mean VEGF expression shows that nonresponsive tumors are more highly immunoreactive and have a significantly greater overall VEGF expression than completely responsive tumors. Of those tumors negative for VEGF, all (100%) were completely responsive to therapy.

In this study, we further investigated whether a variety of frequently used VEGF scoring methods affect the predictive value of the protein. The overwhelming majority of studies use a scoring method based on the 10% cutoff point.[19,20,24,25] Our results demonstrate that VEGF may be predictive of tumor response to preoperative brachytherapy regardless of the scoring system used. However, the selection of the scoring method may have a nonnegligible affect on the final interpretation of the results. More research must be done in the area of scoring methods and how their interpretation may affect the predictive value of the protein.

Although most complete responders are found in the lower end of the distribution of VEGF scores including nearly half with 10% immunoreactivity or less, approximately 26% have more than 80% positive tumor cell staining for VEGF. One explanation for this may lie in the fact that the expression of VEGF is not sufficient for angiogenesis to occur.[7] Numerous antiangiogenic proteins are secreted by tumor cells including endostatin, angiostatin, and thrombospondins whose apoptotic action on endothelial cells counterbalances the effects of proangiogenic agents.[7,26] The 'switch' or imbalance of pro- and antiangiogenic factors leading to tumor angiogenesis may not have yet occurred in these completely responsive yet highly immunoreactive tumors.[6] Similarly, nonresponsive tumors with low VEGF levels may be more antiangiogenic. If so, other mechanisms of radioresistance may be in place in these tumors, such as an imbalance of proliferation versus apoptosis, or deregulated cell-cycle arrest. It may therefore be important to study VEGF in combination with proteins that may have predictive potential such as p53, p27, Bcl-2, or cyclin D and E.[27–31]

In summary, the results of this study indicate that VEGF assessed immunohistochemically from preirradiation tumor biopsies may be a useful marker in the prediction of tumor response to preoperative radiotherapy.

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The Predictive Value of Apoptosis Protease-Activating Factor 1 in Rectal Tumors Treated with Preoperative, High-Dose-Rate Brachytherapy

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BACKGROUND. The objective of this study was to assess the value of apoptosis protease-activating factor 1 (APAF-1) as a predictive marker of response in rectal tumors treated with preoperative, high-dose-rate endorectal brachytherapy.

METHODS. Immunohistochemistry for APAF-1 was performed on 94 rectal tumor biopsy specimens from patients who were treated on a preoperative, high-dose-rate brachytherapy protocol. Tumors were considered positive when > 10% of tumor cells were immunoreactive. The association between APAF-1 expression and tumor response was made using the chi-square test.

RESULTS. Forty-four tumors (43%) were positive for APAF-1. Thirty tumors had complete pathologic tumor regression after preoperative radiotherapy. Of these, 18 tumors were positive for APAF-1. A partial response occurred in 35 tumors. Eighteen tumors (51%) were positive for the protein. Only 8 of 29 nonresponsive tumors (28%) were immunoreactive for APAF-1. A significant association was found between complete tumor regression and positive APAF-1 status ($P = 0.018$). APAF-1 expression in partially responsive tumors was significantly greater than in nonresponsive tumors ($P = 0.03$).

CONCLUSIONS. APAF-1 expression in pretreatment rectal tumor biopsy specimens may be useful as a predictive marker of response to preoperative radiotherapy in patients with rectal carcinoma. *Cancer* **2006;106:284–6.**

KEYWORDS: rectal carcinoma, apoptosis protease-activating factor 1, preoperative radiotherapy, tumor marker.

Apoptosis, or programmed cell death, is an essential process in normal development and tissue homeostasis, because it acts to counter abnormal cell proliferation.[1] The inhibition and deregulation of apoptotic pathways contribute to the pathogenesis of colorectal carcinoma and have been shown to increase tumor resistance to radiotherapy.[2] Tumor cell response to radiation may manifest primarily through the activation of proapoptotic factors, resulting in mitochondria-mediated cell death.[3]

Apoptosis protease-activating factor 1 (APAF-1) is a 130-kD protein that plays a central role in mitochondrial apoptosis.[3] In response to apoptotic stimuli, such as radiation, APAF-1 binds cytochrome *c* and procaspase 9 in the presence of adenosine triphosphate to form a multiproteic complex called the apoptosome.[2] Activation of procaspase 9 by autocatalytic cleavage initiates a cascade of downstream effector caspases, ultimately resulting in apoptosis.[3,4] The objective of this study was to determine whether APAF-1 from pretreatment tu-

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mor biopsies is predictive of response to preoperative radiotherapy in patients with locally advanced rectal tumors.

MATERIALS AND METHODS

Ninety-four patients with rectal adenocarcinoma were entered into the study, and informed written consent was obtained from each participant. Patients were staged according to the International Union against Cancer classification by both endorectal ultrasonography and magnetic resonance imaging (MRI). Patients with abdominal lymph node disease and patients who had distant metastases were excluded from the study. Radiation was delivered preoperatively with an eight-channel endorectal catheter using a high-dose-rate remote after-loading system.[5] A daily fraction of 6.5 grays (Gy) was administered over 4 consecutive days up to a total of 26 Gy. Doses were planned for each patient by using a computed tomography simulator to obtain optimal conformal dosimetry. The dose was prescribed to a clinical target volume that included the macroscopic tumor volume and any intramesorectal deposits visible at MRI. Patients underwent tumor-directed surgery 4–8 weeks after brachytherapy regardless of tumor response.

Pathologic response to preoperative radiotherapy was based on postoperative evaluation of the tumor specimen. A complete tumor response was defined as no histologic evidence of residual viable carcinoma (ypT0); a partial response was determined by the presence of microfoci of residual carcinoma; and a nonresponse was characterized by large areas of residual carcinoma.

Immunohistochemistry

Immunohistochemistry was used to detect the presence of APAF-1 from each of the 94 pretreatment tumor biopsy specimens. Formalin fixed, paraffin embedded sections were cut at a thickness of 3 μm and were dried at 37 °C overnight. Immunohistochemistry was performed using the avidin-biotin complex procedure, including heat-induced epitope-retrieval and enzymatic antigen-retrieval procedures. Incubation with anti-APAF-1 (NCL-APAF-1; Novocastra; 1:100 dilution) was carried out in a moist chamber at 37 °C for 1 hour. Negative controls were treated identically with the primary antibody omitted. Positive controls consisted of normal skin tissue. Immunohistochemistry was evaluated by two independent observers. Tumors were considered positive using the standard > 10% cut-off scoring system.[6] Evaluation of APAF-1 from preirradiation tumor biopsy specimens was performed by evaluators who were blinded to postoperative tumor response.

Statistical Analysis

The association between positive APAF-1 status and tumor response was evaluated by using the chi-square test. Multivariate analysis of patient age, gender, tumor grade, and clinical stage was assessed by response. Statistical analyses were performed using SAS software (version 8.2; SAS Institute, Cary, NC. P values < 0.05 were considered significant.

RESULTS

Clinical staging revealed 3 clinical T2 (cT2) tumors, 3 cT4 tumors, and 88 cT3 tumors. Age, gender, and tumor grade were not associated with tumor response. Of the 94 tumor biopsies, 43% were positive for APAF-1. Thirty tumors had complete pathologic tumor regression after preoperative radiotherapy. Of these, 18 tumors were positive for APAF-1. A partial response occurred in 35 tumors. Eighteen tumors (51%) were positive for the protein. Only 8 of 29 nonresponsive tumors (28%) were immunoreactive for APAF-1. A significant association was found between complete tumor regression and positive APAF-1 status ($P = 0.018$). Similarly, APAF-1 expression in pretreatment tumor biopsies from partially responsive tumors was significantly greater than in nonresponsive tumors ($P = 0.03$).

DISCUSSION

Among the advantages of preoperative radiotherapy for the treatment of locally advanced rectal carcinoma is tumor regression, which generally is carried out by rapid, mitochondria-dependent apoptosis.[1] Complete pathologic tumor regression or a partial tumor response can be achieved in these tumors, increasing the probability of sphincter-sparing procedures.[7] The ability to predict tumor response before treatment using immunohistochemistry for the proteins involved in programmed cell death, such as APAF-1, may provide an additional criterion for the selection of patients for treatment with radiotherapy. The role of APAF-1 has been investigated in melanoma, cervical carcinoma, and other tumor types.[8,9] However, its value as a predictive marker in colorectal carcinoma has yet to be established.

APAF-1 appears to play a crucial role in normal development. APAF-1-deficient mice embryos typically die in utero or shortly after birth and exhibit severe craniofacial abnormalities, retention of interdigital webs, and abnormal eye and inner ear development.[10] APAF-1 knock-out mice show brain overgrowth because of hyperproliferation of neuronal cells.[11] Heterozygous mice do not show these alter-

ations, suggesting that APAF-1 may function as a tumor suppressor gene.[10]

APAF-1 appears to be an essential component of *p53*-mediated apoptosis. Robles et al. identified a classic *p53*-responsive element upstream of the APAF-1 promoter site.[12] When it is bound, *p53* leads to the induction of APAF-1 gene expression. An inverse correlation was found between *p53* mutation and APAF-1 expression in melanoma cell lines.[11] Evidence suggests that the E2F1 transcription factor targets APAF-1 by binding at a site near the APAF-1 promoter region.[13] This activation may lead to disruption of the retinoblastoma pathway, resulting in apoptosis in a *p53*-independent manner.[4]

Previous studies in patients with rectal tumors who were treated with preoperative radiotherapy have investigated the potential use of apoptotic indices (the proportion of tumor cells undergoing apoptosis) from pretreatment biopsies to predict tumor response.[14] Indices of 1–5% appear to correlate significantly with response, whereas nonresponsive tumors have a lower proportion of apoptotic tumor cells (0.5–1.44%).[15,16] Although a higher apoptotic index appears to correspond to a greater likelihood of response, investigators have questioned whether the assessment of apoptosis by terminal deoxyuridine triphosphate nick-end labeling or hematoxylin and eosin processing simply may not be a reflection of the increased proliferation rate of the tumor.[17]

The assessment of APAF-1 in rectal tumors may not necessarily be a direct reflection of the apoptotic state of the cell but, rather, reflects its potential for mitochondria-dependent cell death. Other mechanisms may be influencing APAF-1 expression. For example, Bcl-2 and Bax, located between the inner and outer mitochondrial membrane, function to inhibit and stimulate cytochrome *c* release, respectively. Therefore, it may be important to study APAF-1 expression in relation to other proapoptotic and antiapoptotic proteins.

In the current study, the predictive value of APAF-1 in rectal carcinoma was evaluated. A significant association was found between APAF-1 in pretreatment rectal tumor biopsies and response to preoperative brachytherapy. We conclude that APAF-1 may be a useful predictive marker of response to preoperative radiotherapy in patients with locally advanced rectal tumors.

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A Predictive Model of Rectal Tumor Response to Preoperative Radiotherapy Using Classification and Regression Tree Methods

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Abstract **Purpose:** The ability to predict rectal tumor response to preoperative radiotherapy before treatment would significantly impact patient selection. In this study, classification and regression tree (CART) methods were used to model tumor response to preoperative conformal high-dose rate brachytherapy by assessing the predictive value of vascular endothelial growth factor (VEGF), Bcl-2, p21, p53, and APAF-1.

Experimental Design: Immunohistochemistry was used to detect VEGF, Bcl-2, p21, p53, and APAF-1 from 62 pretreatment rectal tumor biopsies. Scores were assigned as percentages of positive tumor cell staining and were used in CARTanalysis to identify the proteins that best predicted response to radiotherapy. Ten-fold cross-validation was used to prevent overfitting and multiple cross-validation experiments were run to estimate the prediction error.

Results: Postoperative pathologic evaluation of the irradiated tumor bed revealed 43 responsive tumors [20 with complete response (T_0) and 23 with partial response] and 19 nonresponsive tumors. The optimal tree resulting from CARTanalysis had five terminal nodes with a misclassification rate of 18%. Of the five proteins selected for their predictive value, VEGF and Bcl-2 contributed most to the classification of responsive and nonresponsive tumors. All 10 tumors with no VEGF were completely responsive (T_0) to radiotherapy; 85% of those with VEGF and negative for Bcl-2 were responsive to therapy.

Conclusions: VEGF and Bcl-2 status in pretreatment rectal tumor biopsies may be predictive of response to preoperative high-dose rate brachytherapy.

Preoperative radiotherapy for rectal cancer can significantly improve patient survival and reduce local recurrence rates versus postoperative radiation or surgery alone (1–4). Additionally, high-dose-rate preoperative conformal endorectal brachytherapy, a novel therapeutic approach to the treatment of invasive rectal cancer, may result in more frequent tumor down-staging or complete tumor regression, leading to a greater number of sphincter-sparing procedures (5, 6). The ability to predict tumor response before treatment may significantly impact the selection of patients for preoperative radiotherapy as well as potentially modify postoperative treatment plans.

It is now recognized that the differential expression of genes governing cell cycle arrest and apoptosis is an important determinant of radioresponse (7, 8). In normal cells, the *p53* tumor suppressor gene mediates both cell cycle arrest and apoptosis through the transcriptional activation of *p21*, *BCL-2*, and *BAX* among others (9). In response to DNA damage, p53 enhances the transcription of p21, a cyclin-dependent kinase inhibitor that delays the progression of cells from G_1 to S phase of the cell cycle, thereby preventing the replication of damaged DNA (10). p21 has been associated with radiosensitivity and improved outcome in rectal tumors following preoperative radiotherapy (11–13).

Mutations of p53 in rectal cancer have been linked to decreased survival and aggressive malignant behavior (14, 15). Kandioler et al. (16) showed, by DNA sequencing, that p53 mutations were predictive of lower survival rates and decreased response to preoperative radiotherapy. Similar studies using immunohistochemistry to detect p53 protein yield contradicting results (17–20).

p53 may alter angiogenesis by activating vascular endothelial growth factor (VEGF), a potent mediator of new blood vessel formation in tumorigenesis (21, 22). Expression of VEGF is induced by other factors as well, most notably hypoxia (23). *In situ* hybridization studies have found that transcription of VEGF mRNA in rectal tumors is up-regulated during the progression from adenoma to carcinoma (21, 22, 24, 25). Anti-VEGF therapy, in combination with chemotherapy and/or radiotherapy for rectal cancer, is an area of active investigation (26, 27).

Disruption of mitochondrial function and release of cytochrome *c* are early events in the apoptotic cascade (28). In the cytoplasm, cytochrome *c* associates with APAF-1, initiating the downstream cleavage of caspases and eventually resulting in cell death (28, 29). Although little is known about APAF-1 function, loss or mutation of APAF-1 has been associated with radioresistance in several tumor types (30).

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Bcl-2, an antiapoptotic protein inhibiting release of cytochrome *c* and activation of APAF-1, is induced by VEGF and may play a role in determining radioresponse (28-30).

In this study, VEGF, Bcl-2, p21, p53, and APAF-1 in pretreatment rectal biopsies from patients undergoing preoperative conformal high-dose rate brachytherapy (5) were evaluated by immunohistochemistry. Classification and regression tree (CART) methods were then used to assess the value of each protein in predicting tumor response.

Patients and Methods

This study was approved by the Research Ethics Committee of the McGill University Health Center and informed written consent was obtained from 62 patients with rectal adenocarcinoma. Clinical staging according to the International Union against Cancer classification was carried out by both endorectal ultrasonography and magnetic resonance imaging. On the occasion of a disagreement between methods, the highest stage was assigned. Patients with abdominal nodal disease were excluded from the study as were patients with distant metastases. Three patients had cT_2 tumors, one had cT_4, and 58 were cT_3. Radiation was delivered preoperatively with an eight-channel endorectal catheter using a high-dose rate remote after-loading system. A daily fraction of 6.5 Gy was administered over 4 consecutive days to a total of 26 Gy. Each patient was planned using a computed tomography simulator to obtain optimal conformal dosimetry. The dose was prescribed to a clinical target volume that included the gross tumor volume and any intramesorectal deposits visible at magnetic resonance imaging. Patients underwent cancer-directed surgery 4 to 8 weeks after brachytherapy regardless of tumor response.

Tumors were classified as responsive (complete or partial response) or nonresponsive to brachytherapy based on the pathologic evaluation of the specimen postoperatively. Complete response was defined as no histologic evidence of residual viable carcinoma (ypT_0). Partial response was characterized by the presence of at least one microfoci of residual carcinoma. Microfoci ranged from 0.3 to 0.9 cm in diameter. Nonresponsive tumors consisted of larger areas of residual carcinoma, rather than microfoci, that could be identified macroscopically and ranged in size from 2 to 6 cm.

Immunohistochemistry. Immunohistochemistry was used to detect p53, p21, Bcl-2, VEGF, and APAF-1 from pretreatment tumor biopsies. Formalin-fixed, paraffin-embedded serial sections were cut at 3 μm and dried at 37°C overnight. Immunohistochemistry was done using the avidin-biotin complex procedure, including heat-induced epitope retrieval and enzymatic antigen retrieval procedures. Incubation was carried out overnight at 4°C for p21 (clone SX118, 1:100; DAKO, Glostrup, Denmark), Bcl-2 (clone 124, 1:100; DAKO), and VEGF (VEGF-A20, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), and in a moist chamber at 37°C for 1 hour for p53 (clone DO-7, 1:100; DAKO) and APAF-1 (NCL-APAF-1, 1:100; Novocastra, Newcastle, United Kingdom). Negative controls were treated identically with primary antibodies omitted. Positive controls consisted of tissue known to contain the protein of interest. Immunostaining was scored as a percentage of positive tumor cells by two independent observers.

Statistical model. CART methods were used to determine which proteins best predicted response to treatment (31). The CART trees were fit using the *R* statistical software *tree* library package (R Foundation for Statistical Computing, 2004, Vienna, Austria). The best tree fit to the full data has eight terminal nodes (tree not shown) with an overall misclassification rate of 16% (10 of 62).

To assess the amount of overfitting, we did 1,000 10-fold cross-validation experiments (32). In each of those 1,000 experiments, the data set was randomly split into 10 smaller data sets and a pruning method was used to choose the best number of nodes for the original tree pruned with respect to 90% of the data according to the misclassification rate for the other 10% of the data. Although the best average misclassification rate across 1,000 simulations was for five terminal nodes, the difference between five terminal nodes and one terminal node was very small (<1%). With further exploration, we found that average classification rate for one terminal node is primarily due to high variance resampling the small number of patients with zero traces of VEGF in the biopsy. With the reasonably large percentage of responsive tumors in the data set, many resampled data sets consisted primarily of responsive tumors (which made trees with one terminal node competitive with five terminal nodes in terms of misclassification rates).

To resolve the uncertainty in assessing the optimal number of terminal nodes for the full data set, we conducted a two-tailed Fisher's exact test (33) to test for a relationship between the absence/presence of VEGF and response/nonresponse to treatment (Table 1). The *P* value for the Fisher's exact test was <0.03, indicating a significant relationship between absence/presence of VEGF and response/nonresponse to treatment. Because of the instability of the full cross-validation due to the large effect of VEGF but the small number of subjects with negligible VEGF, we removed those 10 observations from the subsequent CART analyses. We fit a new classification tree with the remaining 52 observations and, using 100 10-fold cross-validation experiments, obtained an optimal tree with four terminal nodes. An average cross-validated 22% misclassification rate on the four-node subtree was observed conditioning on positive VEGF levels. We want to emphasize that the best number of terminal nodes for full data set is five and that our subanalysis using Fisher's exact test is merely to confirm that there is strong evidence that VEGF can be used to predict responsiveness to tumors and moderately strong evidence that the remainder of the splits in our five-node tree can improve classification rates beyond that first split.

Results

Postoperative pathologic evaluation of the irradiated tumor bed gave rise to 43 responsive tumors (20 with complete response and 23 with partial response) and 19 nonresponsive tumors. The tumor stage distribution before and after brachytherapy may be found in Table 2. Cytoplasmic immunoreactivity for VEGF, APAF-1, and Bcl-2 ranged from 0% to 100% tumor cell staining. Nuclear immunoreactivity for p53 and p21 varied from 0% to 100% and from 0% to 40% tumor cell staining, respectively.

Of the five proteins initially selected for their potential predictive value, only VEGF, Bcl-2, and p21 contributed to the classification of responsive and nonresponsive tumors (Fig. 1). All 10 tumors with no VEGF immunoreactivity were completely responsive to therapy (ypT_0). Those with >2% VEGF expression were further subdivided by the percentage of positive tumor cell staining for Bcl-2 and p21. A high classification rate was reached for tumors with no Bcl-2 and <92.5% immunostaining for VEGF. Such tumors were responsive to therapy in over 85% of cases

Table 1. Two-way table displaying the deleterious effect of positive VEGF levels on response to treatment

VEGF above 0	Response to treatment		Total
	No	Yes	
Yes	19	33	52
No	0	10	10
Total	19	43	62

NOTE: *P* value for Fisher's exact test of independence < 0.03.

Table 2. Tumor stage distribution before and after brachytherapy

	T_0	T_1	T_2	T_3	T_4	Total
Pretreatment clinical stage (cT)	—	—	3	58	1	62
Postoperative pathologic stage (ypT)	20	11	17	14	0	62

whereas those with greater VEGF levels were largely nonresponsive (71%). Less efficient discrimination was observed in Bcl-2–positive tumors. Of the 10 Bcl-2–positive tumors, 8 had <1.5% tumor cell staining for p21.

Discussion

As tumors grow, their requirement for oxygen and nutrients expands beyond the limit of oxygen diffusion provided by the host vasculature (34). This creates a microenvironment of hypoxia in the central region of the tumor resulting in apoptosis in cells susceptible to low oxygen tension (35). Persistent hypoxic conditions lead to the production of VEGF (29). This cytokine serves as a mitogen for endothelial cells and activates proteolytic enzymes involved in the degradation of the basement membrane as well as the extracellular matrix (27). These processes ultimately result in the growth of a tumor vasculature. The new blood vessels are characterized by increased permeability, causing less efficient delivery of chemotherapeutic agents and decreasing response to radiotherapy (27, 29). Several studies have investigated serum VEGF levels as a prognostic marker in patients with colorectal cancer. A significant association between elevated preoperative serum VEGF and worse prognosis has been reported (36–38).

VEGF has also been shown to act on tumor cells by inducing Bcl-2 (27, 39). Early in the colorectal adenoma-carcinoma sequence, both VEGF and Bcl-2 seem to be up-regulated (25). In invasive cancer, VEGF levels increase whereas Bcl-2 expression may be significantly reduced (25, 40). Bcl-2 could, therefore, be important primarily in sustaining cell survival under initial hypoxic conditions until oxygen and nutrients can be reached via diffusion from newly formed tumor vessels. The presence of VEGF is likely an indirect reflection of the hypoxic state of the tumor.

Of the 10 tumors in this study that had no VEGF, all (100%) were responsive to radiotherapy. Absence of the protein may signify a well-oxygenated tumor that has not yet acquired the need for additional tumor vessels. Vascular permeability and partial oxygen pressure are maintained, thereby enhancing tumor response. Bcl-2–negative tumors with low levels of VEGF may not only be retaining their vascular permeability but might also be more susceptible to radiotherapy due to a lessened antiapoptotic signal. In this study, 85% of tumors with no Bcl-2 and with VEGF <92.5% were responsive to therapy. Nonresponsive Bcl-2–negative tumors with nearly all cells positive for VEGF may no longer require the survival advantage of Bcl-2 provided angiogenesis has already occurred.

Several studies have described both proliferation- and apoptosis-inhibiting roles for p21 (41). Others have reported an association between p21 in pretreatment rectal tumor biopsies and sensitivity to preoperative radiotherapy (11). In our study, p21-negative/bcl-2–positive tumors were largely nonresponsive to treatment (73%); p21-positive/bcl-2–negative tumors were generally associated with responsiveness (71%). However, due to the small number of tumors in our sample, it may be imprudent to draw a conclusion regarding p21 from these data.

There may be several factors confounding the results of this study. First, misclassification of clinical stages using magnetic resonance imaging for rectal cancer has recently been reported as high as 15% for pT_3 tumors (42). More than 95% of patients included in this study were staged by magnetic resonance imaging as cT_3. This may be an overestimation of the true number of T_3 tumors in our sample. The results of this study may prove to be stage dependent. Second, protein expression in biopsies may not be representative of the entire tumor. p21-positive nuclei, for example, cluster and are typically concentrated in the upper one third of the colorectal mucosa. This may possibly be contributing to the inconclusive results involving p21 (43). Third, it is reasonable to assume

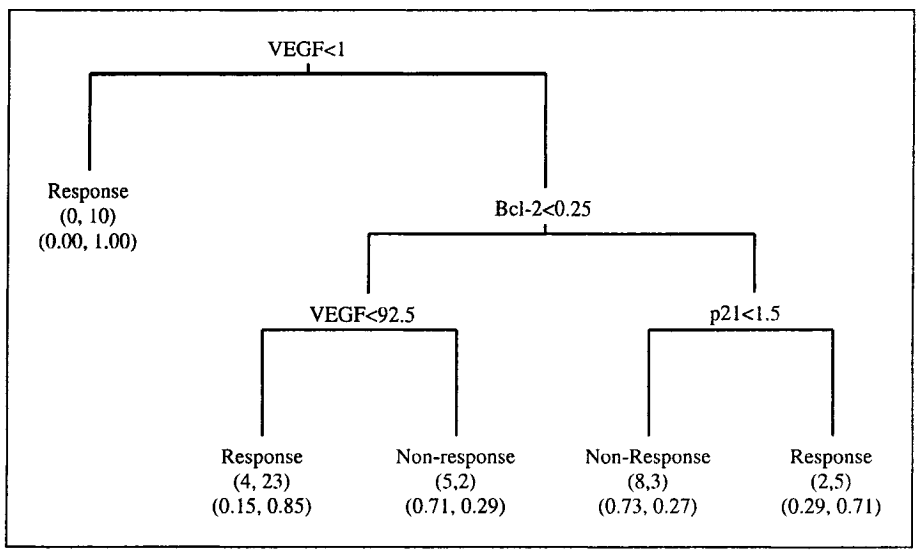

Fig. 1. Optimal tree chosen by cross-validation after preliminary step classifying by the absence or presence of VEGF. The two sets of numbers underneath each terminal node are (number of observed nonresponsive subjects, number of observed responsive subjects) and (proportion of observed nonresponsive subjects, proportion of observed responsive subjects), respectively, for each terminal node.

that the time delay between preoperative brachytherapy and surgery varies between patients. This difference may be affecting the pathologic diagnosis of response/nonresponse in these tumors postoperatively.

Despite these limitations, the results of this study suggest that VEGF and Bcl-2 status in pretreatment biopsies are important in predicting response of invasive rectal tumors to preoperative brachytherapy. Tumors absent for VEGF were associated with complete response to therapy. Those negative for Bcl-2 and with less than maximum immunoreactivity for VEGF were most frequently responsive to radiotherapy (85%).

Whether these results may be upheld across other treatment regimens, such as neoadjuvant radiochemotherapy, remains to be seen. There is evidence to suggest that VEGF, Bcl-2, and p21 may play a role in predicting tumor response to this therapy (44–46). It may, however, be important to tailor the selection of proteins used in the CART to incorporate other potential predictive markers specific to this treatment.

In conclusion, VEGF and Bcl-2 status in pretreatment tumor biopsies may prove to be an additional tool in patient selection for preoperative high-dose rate endorectal brachytherapy. A large-scale prospective study is necessary to validate these preliminary findings.

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Scoring of p53, VEGF, Bcl-2 and APAF-1 immunohistochemistry and interobserver reliability in colorectal cancer

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Molecular tumor markers are often studied in colorectal cancer using immunohistochemistry to determine their prognostic or predictive value. Protein expression is typically assigned a 'positive' score based on a predetermined cutoff. A semiquantitative scoring method that evaluates the percentage of positive tumor cells (0–100%) may provide a better understanding of the prognostic or predictive significance of these markers. The aim of this study was to assess and compare the interobserver agreement of immunohistochemistry scores using a percentage scoring method and three categorical scoring systems. Immunohistochemistry for p53, Bcl-2, vascular endothelial growth factor (VEGF) and apoptotic protease activating factor-1 (APAF-1) was performed on 87 tumor biopsies from patients with rectal carcinoma and scored independently by four pathologists as the percentage of positive tumor cells. Interobserver agreement was assessed by the intraclass correlation coefficient. The intraclass correlation coefficients for p53 and VEGF (>0.6) indicate substantial agreement between observers. The distribution of Bcl-2 and APAF-1 scores in addition to weaker interobserver agreement by percentage scoring suggest that this approach may not be appropriate for these proteins. In conclusion, p53 and VEGF protein expression assessed by immunohistochemistry in colorectal cancer and scored as a percentage of positive tumor cells may be a viable alternative scoring method.
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Although the TNM stage remains the most significant independent prognostic indicator in patients with colorectal cancer, pathologically identical tumors may neither respond to treatment uniformly nor result in similar survival rates.[1] A number of molecular markers involved in proliferation (p53), apoptosis (Bcl-2, APAF-1) and angiogenesis vascular endothelial growth factor (VEGF) are currently being investigated to determine their value as prognostic or predictive factors and in turn their potential for integration into clinical practice.[2–5]

Immunohistochemistry is an indispensable research and diagnostic tool used to assess the presence or absence of molecular tumor markers on paraffin-embedded tissue.[6] Tumor positivity for a given marker is frequently evaluated using predetermined cutoffs such as 10% (≤10% tumor cells staining = negative, >10% = positive).[4,7–10] The employment of categorical scoring systems is motivated by the ease of interpretation of positive tissue by pathologists and is further supported by substantial interobserver agreement. However, it assumes that more detailed analysis of protein expression between 10 and 100%, for example will not contribute any additional relevant information in predicting outcome.[11]

A semiquantitative scoring method that assigns immunohistochemistry scores as a percentage of positive tumor cells (the number of positive tumor cells over the total number of tumor cells) may provide a more complete assessment of protein expression and a clearer understanding of the roles played by potential tumor markers in predicting outcome. Most importantly, by evaluating immunohistochemistry expression semiquantitatively at the

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outset, more relevant cutoffs for tumor positivity may be established for the protein and outcome of interest.

The greatest concern facing such a percentage scoring method is the reproducibility of the scores. In this study, we assess the interobserver agreement of immunohistochemistry scores for four tumor markers known to play a role in progression of colorectal carcinoma and response to radiotherapy namely p53, VEGF, Bcl-2 and APAF-1 and compare the interobserver agreement of percentage scoring to that of three categorical scoring systems.

Materials and methods

In total, 87 pretreatment formalin-fixed paraffin-embedded diagnostic rectal biopsy tissues were collected from a series of patients with rectal adenocarcinoma undergoing preoperative endorectal brachytherapy. [12] Serial sections were cut at 3 μm and immunohistochemistry by the avidin–biotin complex (ABC) procedure, including heat-induced epitope retrieval, was undertaken. Incubation with the primary antibody was carried out in a moist chamber for 1 h at 37°C for p53 (DAKO, clone DO-7, Denmark, 1:100) and at room temperature for VEGF (Santa Cruz Biotechnology, VEGF-A20, USA, 1:100) and APAF-1 (Novocastra, NCL-APAF-1, 1:40). Overnight incubation at 4°C was performed for Bcl-2 (DAKO, clone 124, Denmark, 1:100). Negative controls were treated identically with the primary antibodies omitted. Positive controls consisted of tissue known to contain the protein of interest.

Nuclear positivity for p53 and cytoplasmic positivity for VEGF, Bcl-2 and APAF-1 were evaluated only in areas of invasive carcinoma. Immunoreactivity was scored as the number of positive tumor cells over total tumor cells, independently by four pathologists (CCC, JRJ, RPM, AL); in general each slide took on average 30 s or less to score. No specific instructions or illustrations were presented to pathologists to assist in their evaluation. Percentage scores were subsequently categorized using the 0% cutoff (0% staining *vs* any staining), the 10% cutoff ($\leq$10% tumor cell staining *vs* >10% staining) and a three-category scoring system consisting of 0% staining, between 1 and 50% staining and >50% staining.

Statistical Analysis

The interobserver agreement for the 0, 10 and 0, 1–50, >50% cutoff scoring systems were evaluated using Light's Kappa coefficient.[13] The Kappa coefficient (k) is a useful measure of agreement for categorical data as it takes into account the probability that observers achieved the same scores by chance. General guidelines for the interpretation of Kappa suggest that values between 0.81 and 1.0 should represent 'almost perfect' agreement, 0.61–0.80 'substantial' agreement, 0.41–0.60 'moderate' agreement, 0.21–0.40 'fair' agreement, and 0–0.20 'slight' agreement.[14]

The intraclass correlation coefficient is the most commonly used method to assess interobserver agreement for quantitative measurements.[15] Similar to the simple Pearson correlation coefficient that measures association, the intraclass correlation coefficient additionally estimates agreement between scores from different observers on the same patients. The closer the intraclass correlation coefficient is to 1, the better the agreement between observers. The intraclass correlation coefficient was employed to assess interobserver agreement of percentage scores.

Although no recommendations for the interpretation of the intraclass correlation coefficient have been detailed, reports in the literature have supported the use of the following guidelines: a coefficient of reliability >0.75 indicates 'strong' agreement, between 0.4 and 0.75, 'good' agreement, and <0.4, 'poor' agreement.[16] It has also been suggested that the values for the Kappa coefficients may be equivalent to the intraclass correlation coefficient making their direct comparison appropriate.[17]

Confidence intervals (95%) were found by 10 000 bootstrap replications of the dataset. All analyses were carried out using SAS Version 8.2 (The SAS System, NC, USA).

Results

p53

Overall mean p53 protein expression was 37% (Table 1). Approximately 72% of tumors were positive for the protein. The frequency distribution of p53 scores was nearly uniform above 0% (Figure 1). The reproducibility of p53 scores was

Table 1 Mean and standard deviation of scores (%) for pathologists 1–4 and overall mean protein expression

	Overall	*1*	*2*	*3*	*4*
p53	36.90±34.09	34.07±33.90	34.43±29.61	32.36±28.67	46.71±41.27
VEGF	45.15±37.69	51.96±39.07	39.26±34.43	31.11±11.03	58.58±39.93
Bcl-2	9.47±22.98	14.16±28.02	9.27±22.33	4.14±13.46	10.06±24.48
APAF-1	17.70±32.21	29.22±39.27	14.85±26.21	2.6±7.99	23.97±38.36

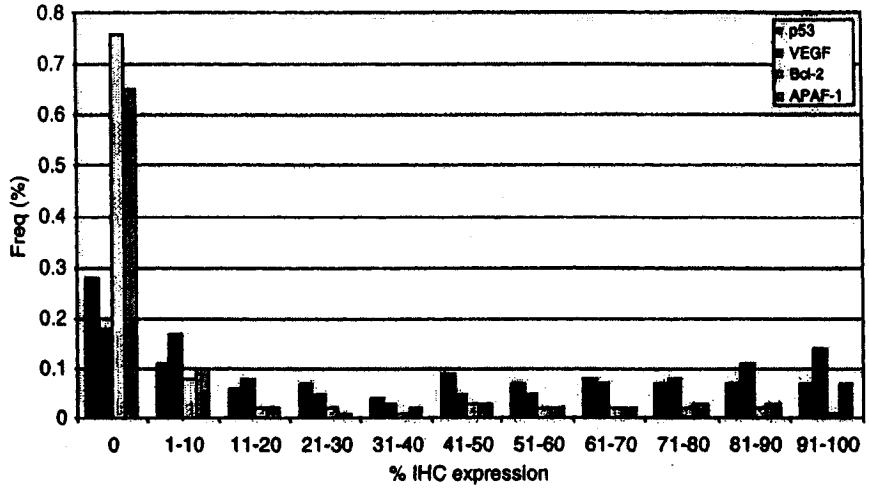


Figure 1 Distribution of p53, VEGF, Bcl-2 and APAF-1 scores.

Table 2 Intraclass correlation coefficient measuring agreement between percentage scores and Kappa coefficients (*k*) measuring agreement of scores using the 0% cutoff, 10% cutoff and 0, 1–50, >50% cutoffs. Intervals represent 95% confidence intervals

	N	*Intraclass correlation coefficient*	*k (0% cutoff)*	*k (10% cutoff)*	*k (0, 1–50, >50% cutoffs)*
p53	86	0.755 (0.67, 0.82)	0.831 (0.73, 0.92)	0.740 (0.63, 0.84)	0.588 (0.48, 0.68)
VEGF	87	0.624 (0.52, 0.71)	0.565 (0.39, 0.71)	0.569 (0.45, 0.68)	0.434 (0.33, 0.53)
Bcl-2	79	0.533 (0.34, 0.69)	0.561 (0.43, 0.68)	0.490 (0.33, 0.63)	0.407 (0.26, 0.55)
APAF-1	85	0.497 (0.41, 0.58)	0.514 (0.40, 0.62)	0.434 (0.33, 0.53)	0.377 (0.30, 0.45)

substantial for both percentage scoring and the 10% cutoff (intraclass correlation coefficient = 0.755 and $k = 0.740$, respectively) (Table 2). Excellent agreement was achieved when no positivity (0%) *vs* any positivity was evaluated ($k = 0.831$). The 0, 1–50, >50% scoring method produced the least amount of agreement between observers. p53 staining was evaluated with less difficulty when no nuclei or nearly all nuclei were positive for the protein (Figure 2a). Staining intensity was generally moderate to strong. Positivity was confined to tumor cell nuclei in the majority of cases. Both the presence of cytoplasmic positivity (Figure 2b) and weak staining intensity in nuclei were largely responsible for the variation in scores.

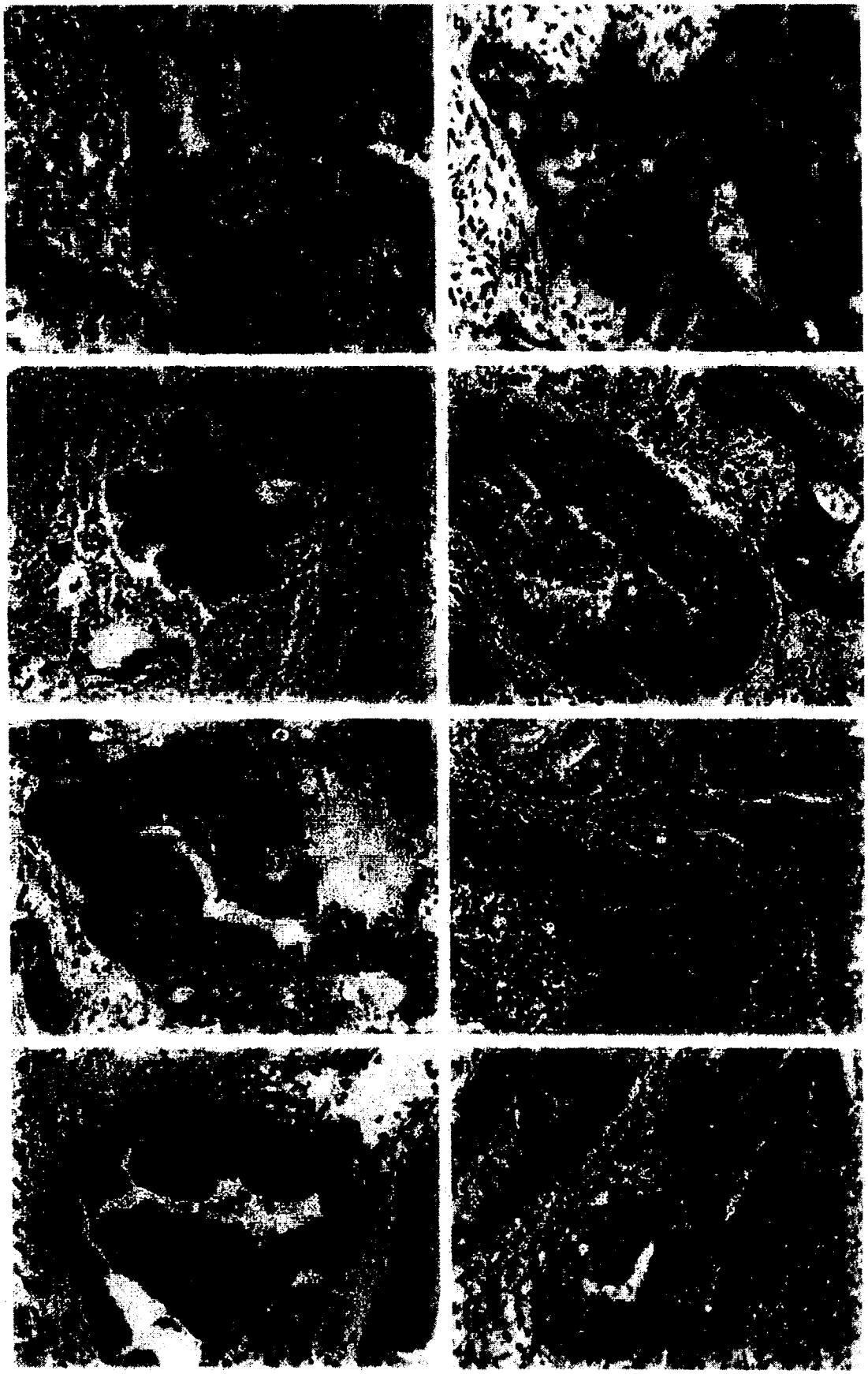
VEGF

The distribution of VEGF scores was U-shaped (Figure 1) with an overall mean cytoplasmic expression of 45% (Table 1). The intraclass correlation coefficient for percentage scoring was 0.624 reflecting a substantial degree of interobserver agreement (Table 2). The categorical scoring systems yielded moderate agreement between observers, the least reproducible being the 0, 1–50, >50% method. The intensity of staining for VEGF varied from weak to strong (Figure 2c). Considerable disagreement between scores could be attributed to weakly stained tumor cells. Infiltration of tumors with a large number of neutrophils may have contributed to the overestimation of the number of positive tumor cells (Figure 2d).

Bcl-2

Approximately 76% of tumors demonstrated complete absence of Bcl-2 (Figure 1). Mean Bcl-2 expression was less than 10% (Table 1). Moderate interobserver agreement was found for percentage scoring as well as for the 0 and 10% cutoffs (Table 2). Agreement was weakest for the 0, 1–50, >50% scoring method ($k = 0.407$). Staining intensity was the primary cause of disagreement of scores between pathologists. Although lymphocytes reacted strongly with the Bcl-2 antibody, only weak to

Figure 2 p53 (**a**, **b**), VEGF (**c**, **d**), Bcl-2 (**e**, **f**) and APAF-1 (**g**, **h**) staining. Tumors in (**a**, **c**, **e** and **g**) resulted in a high degree of interobserver agreement whereas those in (**b**, **d**, **f** and **h**) lead to low interobserver agreement.



moderate staining was found in tumors expressing the protein (Figure 2e). Infiltration of tumors with large numbers of lymphocytes may have also contributed to disagreement in percentage scores (Figure 2f).

APAF-1

Mean APAF-1 expression determined by each of the four pathologists varied significantly from 2.6 to 29% (Table 1). Approximately 64% of tumors were completely negative for the protein (Figure 1). Moderate agreement was achieved for percentage scoring, as well as for the 0 and 10% cutoffs. The strongest agreement was produced when no staining (0%) *vs* any positive staining was evaluated ($k=0.514$). APAF-1 positivity was strong in neutrophils and normal mucosa but only weak to moderate staining occurred in tumors expressing the protein (Figure 2g). Substantial neutrophilic infiltration in tumors may have led to disagreement between observers (Figure 2h).

Discussion

The usefulness of any immunohistochemistry scoring method is limited not only to its ability to optimize the prognostic or predictive value of tumor markers but also to its reproducibility. Studies on interobserver agreement in colorectal carcinoma are uncommon. Several studies using the 10% cutoff scoring method describe a high degree of concordance between pathologists evaluating positive and negative tumors.[18–20] This type of agreement typically overestimates true categorical agreement by ignoring the probability that scores were obtained by chance, an important consideration when scores are not evenly distributed as was seen for Bcl-2 and APAF-1 in this study.[21]

The reproducibility of p53 scores either as percentages or by way of the 10% cutoff scoring method was high. Although agreement was strongest at the 0% cutoff, the distribution of p53 expression suggests that it may be important to evaluate the complete range of scores.

The interobserver agreement of percentage scores for VEGF in this study was higher than those for the 0 and 10% cutoffs. The distribution of VEGF scores indicates that percentage scoring may provide additional information about the protein that would otherwise go unrecognized by categorizing positivity according to predetermined cutoffs. We recently demonstrated in patients with rectal cancer undergoing preoperative radiotherapy that mean VEGF expression was significantly higher (63%) in biopsies from patients with nonresponsive tumors than from tumors with complete pathologic response (37%) (P-value $=0.0035$) hence exemplifying the use of percentage scores.[22]

The reproducibility of Bcl-2 percentage scores was similar to the 10% cutoff. The greatest interobserver agreement was found using the 0% cutoff. Approximately 76% of tumors in this study were completely negative for the protein. This result is in line with the literature which states that the frequency of Bcl-2 expression in rectal carcinoma is less than 30%.[23] Kim *et al*[23] demonstrated that the rate of Bcl-2 overexpression decreases with more advanced Dukes stage. In this study, 98% of rectal biopsies were taken from patients with clinically diagnosed cT3 tumors. This may have biased our results in favor of the 0% cutoff and against percentage scoring as overexpression of Bcl-2 would not be expected to vary significantly in this sample. The interobserver agreement of percentage scores may be better assessed in colorectal adenomas known to frequently overexpress the protein.[23] Our results show that Bcl-2 expression scored as 0% positive tumor cells *vs* any tumor cell staining leads to the highest degree of interobserver agreement in rectal tumors of the same stage.

Recent evidence suggests that *APAF-1* may function as a tumor-suppressor gene.[24] Loss of tumor suppression leads to loss of wild-type APAF-1 protein translating into absence of staining via immunohistochemistry. It is therefore reasonable to suggest that the 0% scoring method with the highest degree of interobserver agreement may be a more meaningful method of evaluation than scoring by percentages for this protein. Although p53 acts as a tumor-suppressor gene as well a similar argument against percentage scoring cannot be used.[25] The short half-life of wild-type p53 renders the protein undetectable to immunohistochemistry.[26] Immunohistochemistry for mutant p53 is based on the assumption that the abnormal protein cannot act as a transcriptional factor hence accumulating in the cell.[25] A comparison or DNA sequencing analysis and immunohistochemistry to detect mutant p53 has revealed a significant false-positive rate for the latter.[25] Immunostaining with p53 antibodies appears therefore to detect abnormal accumulation of p53 in the cell and is not limited to detection of the mutant protein. It is possible that p53 scores evaluated as the percentage of abnormal accumulation of p53 will prove to be a useful predictive factor.

Percentage scoring should allow a more thorough assessment of the predictive or prognostic significance of tumor markers. The correlation between the immunohistochemistry expressions of several proteins can be assessed. Pich *et al*[27] performed percentage scoring of Ki-67, PCNA and MIB-1 expression in non-Hodgkin lymphoma. They found a strong linear correlation for all proteins and used this finding to argue that Ki-67, PCNA and MIB-1 labeling were reliable and complementary methods to assess the proliferative activity of intermediate grade non-Hodgkin lymphoma. By studying the mean expression of Ki-67, PCNA and MIB-1, they identified subtypes of intermediate grade non-

Hodgkin's lymphoma with potentially different prognoses.

Logistic regression is often used to select predictive factors from a pool of possible tumor, host or treatment variables. The risk of development of cancer using serum tumor markers (such as CEA), or the probability of local tumor control with varying doses of radiation are examples of logistic regression with quantitative variables to predict outcome.[26,29] Percentage scoring of immunohistochemistry can be applied similarly to determine how the odds of a binary outcome (response/no response to treatment) change with increases or decreases in protein expression.

Finally, by first quantifying scores, other statistical approaches such as receiver operating characteristic (ROC) analysis can be used to determine the sensitivity and specificity of tumor markers as well as the optimal cutoffs for positivity.[28] By percentage scoring we have shown how classification and regression tree (CART) methods could be used to select proteins playing a role in predicting rectal tumor response to preoperative radiotherapy and to determine the protein cutoff values for optimal discrimination between responsive and nonresponsive tumors.[30]

Percentage scoring of immunohistochemistry expression in colorectal tumors may be suitable for proteins that exhibit a wide range of tumor cell positivity with moderate to strong staining intensity and a high degree of interobserver agreement. The results of this preliminary study on the interobserver agreement of percentage scoring demonstrate that the evaluation of p53 and VEGF using this approach appears to be a reproducible method and viable alternative for the evaluation of immunohistochemistry.

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