Prognostic biomarkers in squamous cell carcinoma of the anus: a systematic review

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BACKGROUND: Recent decades have seen combination chemoradiotherapy become the standard treatment for anal squamous cell carcinoma (SCC). However, the burden of this disease continues to rise, with only 10% of patients with metastatic disease surviving >2 years. Further insight into tumour characteristics and molecular biology may identify novel therapeutic targets. This systematic review examines current prognostic markers in SCC of the anus.

METHODS: An extensive literature search was performed to identify studies reporting on biomarkers in anal cancer in the context of clinical outcome following treatment primarily with chemoradiotherapy.

RESULTS: In all, 21 studies were included. A total of 29 biomarkers were studied belonging to 9 different functional classes. Of these biomarkers, 13 were found to have an association with outcome in at least one study. The tumour-suppressor genes p53 and p21 were the only markers shown to be of prognostic value in more than one study.

CONCLUSIONS: An array of biomarkers have been identified that correlate with survival following chemoradiotherapy in anal cancer. However, investigators are yet to identify a biomarker that has the ability to consistently predict outcome in this disease. Further studies are needed to elucidate whether these candidate biomarkers demonstrate their optimum value when they serve as targets for new therapeutic strategies.

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Anal cancer is a disease whose incidence has risen markedly in several parts of the world including Europe and the United States (Rousseau et al, 2005). Of notable concern is the significant impact of this disease on the young male population, with incidence rates of up to 37 per 100 000 in homosexual men (Place et al, 2001). Of men who have sex with men (MSM), 95% are seropositive for the implicated viral pathogen, human papilloma virus (subtypes 16, 18, 32 and 34) (Palefsky et al, 2005). Also, 81% of MSM have anal intraepithelial neoplasia (AIN; premalignant lesion) (Palefsky et al, 2005). HPV is associated with the proteins E6 and E7 that can silence important tumour-suppressor proteins in normal cells (Mammas et al, 2008). These proteins, namely p53 and Rb, normally exert apoptotic cellular effects to prevent disordered cell growth (Mammas et al, 2008). Removal of this growth restriction may lead to malignant transformation (Mammas et al, 2008). At the time of diagnosis, up to 50% of patients have locoregional disease with visceral metastases present in 10% of patients (UKCCCR Anal Cancer Trial Working Party, 1996; Rousseau et al, 2005). The most common sites are the liver and lungs (Rousseau et al, 2005).

In the past, the mainstay of treatment for anal carcinoma was abdominoperineal resection with formation of a permanent end colostomy. Over the last two decades, combined chemoradiotherapy pioneered in the 1970s by Nigro et al (1974) has become the standard of care with salvage abdominoperineal resection reserved for a small proportion of cases refractory to chemoradiotherapy (Place et al, 2001). Despite respectable treatment response rates in non-metastatic disease with overall survival rates of 60–75%, only 10% of patients with distant metastases survive 2 years from the time of their diagnosis (Cummings, 2006; Das et al, 2008). Furthermore, it is evident that treatment response shows a degree of heterogeneity between individual patients within a specific stage category, suggesting a spectrum of chemoradiation sensitivity. Furthermore, the acute and long-term toxicity associated with standard chemoradiation schedules are considerable, and therefore predictors of response and novel therapeutic approaches are required to allow treatments to be tailored according to treatment sensitivity.

Clinical and pathological factors have been evaluated extensively with regard to outcomes in anal cancer. Clinicopathological parameters have not consistently enabled prediction of response to currently available treatment modalities. This had led to considerable interest in prognostic and predictive biomarkers as putative means to improving patient survival. A prognostic biomarker gives information regarding the patient’s overall outcome, irrespective of the therapy received, whereas a predictive biomarker provides information about the effect of a specific therapeutic intervention (Oldenhuis et al, 2008). Molecular analysis may permit the tailoring of treatments to individual
patients and help identify new therapeutic targets. Tyrosine kinases (TKs), which have an established role in malignant transformation of human cells, have served as major target in cancer treatment strategies such as imatinib, the BCR-ABL TK inhibitor used in the treatment of chronic myeloid leukemia and GISTs (Madhusudan and Ganesan, 2004). In recent years, the epidermal growth factor receptor (EGFR – particularly HER-1 and HER-2) has been the most extensively investigated TKs and now forms a significant component of the ongoing research into molecular targeted cancer therapy. In non-small cell lung cancer, mutations in the TK domain of the EGFR have been shown to be associated with a high treatment response rate to the EGFR TK inhibitor erlotinib (Paz-Ares et al, 2006). Randomised trials in metastatic colorectal cancer have demonstrated that use of monoclonal antibodies directed against EGFR (HER-1), namely cetuximab and panitumumab, is associated with favourable outcomes in patients expressing the wild-type form of K-ras proto-oncogene (Siddiqui and Piperdi, 2009). Therefore, a search for putative predictive markers in anal cancer is warranted in order to explore whether these and other targeted treatments should be assessed in the setting of early and/or metastatic anal cancer.

The purpose of this systematic review was to evaluate the literature available to-date on biological and molecular prognostic factors in squamous cell carcinoma (SCC) of the anus.

MATERIALS AND METHODS

A systematic review was undertaken to identify the prognostic significance of biomarkers in anal cancer. During this process, reference to the AMSTAR measurement tool was made (Shea et al, 2007). This 11-component assessment tool was constructed to assess the methodological quality of systematic reviews. Although the reproducibility and construct validity of this tool is currently uncertain, preliminary findings suggest that AMSTAR has good construct validity and is a reliable tool for the methodological assessment of systematic reviews.

A comprehensive literature search was performed by two independent reviewers (TL and DK) using the PubMed, Web of Science, Embase and Google Scholar databases. This search was conducted up to 9 January 2010. The following keywords were used in various combinations: 'anal cancer', 'biomarkers', 'molecular markers', 'biological markers', 'prognosis' and 'clinical outcome' in addition to searching for individual biomarkers. Further relevant studies were identified by searching the references cited in identified publications and also by utilisation of the PubMed 'related articles' tool (Shalhoub et al, 2009).

No exclusions from the analysis were made based on the date of the publication or the type of the study. Articles not published in the English language were excluded. Studies were excluded because of the absence of (1) any data regarding clinical outcome or prognosis; (2) potentially extractable or a complete set of data considered necessary; (3) peer review status; and (4) the use of chemotherapy/radiotherapy as a primary treatment. Studies were also excluded if they referred to AIN rather than carcinoma.

In addition to patient demographics and tumour characteristics, outcomes of interest related to the study of potential biomarkers included the type of treatment received, tumour recurrence and patient relapse rates, disease-free survival (DFS), overall survival and mortality.

RESULTS

Using the search methods described above, a total of 179 articles were identified. Of these, 73 were initially excluded as they were not studies of anal cancer. Eight articles were not related to the study of biomarkers. A further 30 articles were also excluded by abstract review; 13 of these were review articles and 17 were case reports. Of the remaining 68 references searched in full, 11 studies were excluded as they included the premalignant form, AIN, with no discrimination of results between the two disease groups. Another 17 studies were excluded as they combined data from human cancers of different anatomical locations (including anal carcinoma) with no breakdown of results for the various cancer types. Sixteen studies of biomarkers in anal cancer were excluded as correlation with prognosis was not assessed. Finally, three studies failed to meet the inclusion criteria, as the primary form of treatment in these studies was radical surgery rather than chemoradiation. Ultimately, 21 studies remained for full inclusion in this literature review (Figure 1) (Fontana et al, 1991; Tanum et al, 1992; Goldman et al, 1993; Holm and Tanum, 1996; Tanum and Holm, 1996; Allal et al, 1998, 2003, 2004; Grabenbauer et al, 1998; Bonin et al, 1999; Indinnimeo et al, 1999, 2000a, b, 2001; Wong et al, 1999; Holm et al, 2001; Le et al, 2005; Alvarez et al, 2006; Nilsson et al, 2006; Brueland et al, 2008; Ajani et al, 2009).

The 21 studies included in the analysis evaluated a number of different of biomarkers, which are discussed below and illustrated in Figure 2.

TUMOUR-SUPPRESSOR GENES

The primary role of tumour-suppressor genes is to regulate cell division, differentiation and apoptosis in order to prevent genetic damage that may lead to the development of cancerous cells. p53, p21, p27, p16 and the retinoblastoma (RB) gene are examples of such genes whose prognostic significance has been studied in anal carcinoma.

p53 The p53 gene located on chromosome 17p13.1 encodes a protein that has an important role in cell cycle regulation and apoptosis (Kastan et al, 1995). Additionally, wild-type p53 has been linked with cellular DNA damage repair and inhibition of angiogenesis (Vogelstein et al, 2000). This gene has been extensively studied in several human cancers, and mutations of this gene are considered to be the commonest genetic anomaly in human tumours (Wong et al, 1999). Evidence suggests that mutated p53 can lead to genomic instability and permit malignant transformation of previously non-cancerous cells (Finlay et al, 1989).
Eight studies evaluated the prognostic significance of p53 in anal carcinoma (Table 1) (Tanum and Holm, 1996; Bonin et al, 1999; Indinnimeo et al, 1999; Wong et al, 1999; Allal et al, 2003; Le et al, 2005; Nilsson et al, 2006; Ajani et al, 2009). Immunohistochemistry was utilised in all studies to assess nuclear p53 status in anal carcinoma paraffin-embedded specimens. Wild-type p53 protein has a relatively short half-life and is less readily detectable by immunohistochemical staining. However, mutated p53 typically undergoes a conformational change that stabilises the protein, resulting in nuclear accumulation and therefore positive staining (Neal et al, 2006). In these studies, p53 was considered to be overexpressed if >5% of tumour cells stained positive for nuclear p53. The proportion of patients with anal carcinoma overexpressing p53 ranged from 34 to 100%.

Two studies demonstrated a significant correlation between p53 status and clinical outcome in anal carcinoma. Allal et al (2003) found through multivariate analysis that patients with p53-positive tumours had a lower rate of locoregional control (RR, 0.38; \( P = 0.03 \)) and shorter DFS (RR, 0.29; \( P = 0.003 \)). Similarly, Wong et al (1999) showed that p53 overexpression was an independent adverse marker for DFS in patients with anal carcinoma treated with combined chemoradiotherapy (\( P = 0.01 \)). Another study consisting of 64 patients reported a trend using univariate analysis towards higher locoregional failure rates following chemoradiotherapy in patients with mutant p53 compared with those with the wild-type form (48 vs 27%, \( P = 0.14 \)) (Bonin et al, 1999). However, this finding did not reach statistical significance and no further correlation was seen with either DFS or overall survival (Bonin et al, 1999). The five remaining studies examining p53 did not identify any correlation between its overexpression and prognosis.

**Table 1** Studies of tumour-suppressor genes (p53) in anal carcinoma.

| Authors         | Year | Biomarker | Patients | Method  | Radio ± chemo-therapy (n) | Primary surgical treatment (n) | Mean (median) follow-up period in months | Biomarker +ve patients | Survival (biomarker +ve vs -ve patients) | Correlation with prognosis |
|-----------------|------|-----------|----------|---------|---------------------------|---------------------------------|------------------------------------------|------------------------|-------------------------------------------|-----------------------------|
| Tanum and Holm  | 1996 | p53       | 97       | IHC     | 97 chemo-radiotherapy      | 0                               | 60                                       | 34%                    | Not reported                              | No prognostic significance identified |
| Bonin et al     | 1999 | p53       | 64       | IHC     | 64 chemo-radiotherapy      | 0                               | (57)                                    | 48%                    | 48 vs 33% DFS                              | No prognostic significance identified |
| Indinnimeo et al| 1999 | p53       | 14       | IHC     | Not reported               | Not reported                     | 57.6                                    | 60%                    | 44 vs 40% DFS                              | No prognostic significance identified |
| Wong et al      | 1999 | p53       | 49       | IHC     | 49 chemo-radiotherapy      | 0                               | (54)                                    | 51%                    | 62 vs 67% 5-year DFS                       | p53 expression associated with reduced DFS (\( P = 0.01 \)) |
| Allal et al     | 2003 | p53       | 98       | IHC     | 47 radiotherapy alone 51 chemo-radiotherapy | 0 | (124)                                    | 43%                    | No prognostic significance identified |
| Le et al        | 2005 | p53       | 21       | IHC     | 21 chemo-radiotherapy      | 0                               | Not reported                            | 100%                   | Not reported                               | No prognostic significance identified |
| Nilsson et al   | 2006 | p53       | 214      | IHC     | Not reported               | 7 local excision, 6 APR          | Not reported                           | 50%                    | Not reported                               | No prognostic significance identified |
| Ajani et al     | 2009 | p53       | 30       | IHC     | 30 chemo-radiotherapy      | 0                               | Not reported                           | RR = 1.22 for DFS |                               | No prognostic significance identified |

Abbreviations: APR = abdominoperineal resection; DFS = disease-free survival; IHC = immunohistochemistry; Rb = retinoblastoma; RR = relative risk. \(^a\)Patients included in final biomarker analysis. \(^b\)In studies where patients have been differentiated by the presence or absence of tumour biomarker overexpression, overexpression is considered as biomarker positive.
are predominantly induced by p53 and it is considered to be a mediator of the tumour-suppressor activity of p53 (el-Deiry et al., 1993). However, it also appears to function via mechanisms independent of the p53 gene in response to cellular DNA damage (Roninson, 2002). Underexpression of p21 has been associated with poor prognostic outcome in various human cancers including SCC of the lung (Komiya et al., 1997).

Three studies have assessed the relationship between p21 and prognosis in anal carcinoma using immunohistochemistry to determine the level of p21 expression (Table 2) (Holm et al., 2001; Allal et al., 2004; Nilsson et al., 2006). The study by Holm et al. (2001) included 94 patients with anal carcinoma. They reported high p21 expression in 71% of patients with anal carcinoma (vs 10% positivity in negative controls of normal anal squamous epithelium). Univariate analysis demonstrated a significant association between lack of p21 immunoreactivity and poorly differentiated carcinomas (P = 0.004) and shorter overall survival (P = 0.013) (Holm et al., 2001). No significant correlation was seen between p21 expression and tumour stage or presence of nodal metastases in this study (Holm et al., 2001). Nilsson et al (2006) and Allal et al. (2004) reported high p21 expression in 69 and 65% of anal cancers, respectively. Both studies demonstrated a trend towards shorter survival in p21-negative tumours, but statistical significance was not reached (P = 0.08 and P = 0.1, respectively). Conversely, multivariate analysis by Nilsson et al. (2006) found that patients with tumours underexpressing p21 had a significantly higher locoregional failure rate (P < 0.05).

p16 The p16 protein, also known as cyclin-dependent kinase inhibitor 2A (CDKN2A), specifically targets and inhibits the activity of CDK 4 and CDK 6, both of which are involved in cell proliferation (Bruland et al., 2008). Studies of p16 have demonstrated a potential prognostic role for this biomarker in oropharyngeal SCC (Shi et al., 2009). Two recent studies consisting of 55 and 30 patients, respectively, investigated the possible role of p33 in predicting outcome in anal cancer (Table 2) (Bruland et al., 2009; Ajani et al., 2009). Neither study identified any association between p16 protein expression and clinical outcome or treatment response (Bruland et al., 2008; Ajani et al., 2009).

The RB gene The RB gene, a tumour-suppressor gene originally found to be mutated in the rare paediatric malignancy, retinoblastoma, encodes a nuclear protein involved in cell differentiation, proliferation and apoptosis (Du and Searle, 2009). RB gene mutations leading to loss of its tumour-suppressor function has been identified in a variety of human malignancies (Bourgo et al., 2009; Du and Searle, 2009). One study of the RB gene in anal carcinomas found no significant rearrangement or loss of the RB locus in malignant (or benign) tissue (Crook et al., 1991).

Tanum and Holm (1996) evaluated 97 patients treated with chemoradiotherapy for anal carcinoma (Table 2). Of the pretreatment tumour specimens, 95% stained positive for the RB protein. No relationship was identified between protein expression and the clinical course of the disease.

EGFR Four transmembrane TK proteins (HER-1 to HER-4) make up the EGFR family of receptors that regulate cellular proliferation, angiogenesis, apoptosis and migration via binding of specific ligands (Neal et al., 2006; Coate et al., 2009). The intimate association between the EGFR overexpression and cancer development has served as a platform for the introduction of new therapies targeting these receptors. This is therapeutically achieved through direct inhibition of TK or through antibody-mediated targeting of receptors. Monoclonal antibodies directly bind to the external domain of these TK receptors. Traztuzumab (Herceptin), a monoclonal antibody against the HER-2 receptor, has been widely established in breast cancer patients overexpressing HER-2 with significantly improved survival rates reported in these
patients (Treish et al, 2000). As previously mentioned, the TK inhibitor erlotinib has been successfully used in the treatment of non-small cell lung cancer (Paz-Ares et al, 2006).

Tanum and Holm (1996) studied HER-2 protein expression in 97 patients with anal carcinoma who received chemoradiotherapy. HER-2 protein expression was undetectable (Table 3) (Tanum and Holm, 1996). Similarly, in a Canadian study of 21 patients with anal cancer, all samples were negative for HER-2 (Le et al, 1996). Similarly, in a Canadian study of 21 patients with anal cancer, all samples were negative for HER-2 (Le et al, 1996). Similarly, in a Canadian study of 21 patients with anal cancer, all samples were negative for HER-2 (Le et al, 1996). Similarly, in a Canadian study of 21 patients with anal cancer, all samples were negative for HER-2 (Le et al, 1996). Similarly, in a Canadian study of 21 patients with anal cancer, all samples were negative for HER-2 (Le et al, 1996).

However, this study found that EGFR (HER-1) was strongly expressed in 100% of patients (Le et al, 2005). Ajani et al (2009) also performed immunohistochemical analysis of EGFR and reported expression in 86% of patients with anal cancer, but no significant correlation was observed between the degree of EGFR staining and DFS. One study detected EGFR using immunohistochemistry in 55% of anal SCCs. They also performed fluorescent in situ hybridisation (FISH) to assess EGFR gene copy numbers (Alvarez et al, 2006). There was no correlation between EGFR status and clinicopathological parameters. Furthermore, there was a lack of concordance between the two detection techniques (Alvarez et al, 2006).

Regulators of apoptosis

Apoptosis and the genes that regulate it exert a profound effect on the malignant phenotype in mammalian cells (Lowe and Lin, 2000) (Table 4). Mutations in genes encoding these apoptotic regulatory proteins can lead to tumour initiation, progression or metastasis (Lowe and Lin, 2000).

### Nuclear factor-κB (NF-κB)

The nuclear transcription factor NF-κB has been shown to prevent apoptosis through direct inhibitory mechanisms as well as indirectly by blocking mitochondrial-mediated apoptosis through neutralisation of reactive oxygen species (Naugler and Karin, 2008). Moreover, poor chemotherapeutic responses have been attributed to NF-κB-induced resistance to apoptosis (Naugler and Karin, 2008).

A recent study (Ajani et al, 2009) of 30 patients with anal cancer treated with chemoradiotherapy reported that NF-κB was an independent predictor of DFS. Multivariate analysis revealed that patients with higher NF-κB levels, detected by immunohistochemistry, had shorter DFS (P = 0.002) (Ajani et al, 2009).

### Regulators of apoptosis

| Authors | Year | Biomarker | Patients (n) | Method | Radio ± chemo-therapy (n) | Primary surgical treatment (n) | Mean (median) follow-up period in months | Biomarker +ve patients | Survival (biomarker +ve vs –ve patients) | Correlation with prognosis |
|---------|------|-----------|-------------|--------|--------------------------|-------------------------------|----------------------------------------|------------------------|--------------------------------------|--------------------------|
| Tanum and Holm | 1996 | c-erb B-2 | 97 | IHC | 97 chemo-radiotherapy | 0 | 60 | 0 | Not reported | No prognostic significance identified |
| Le et al | 2005 | HER-2 | 21 | IHC | 21 chemo-radiotherapy | 0 | Not reported | 0 | Not reported | No prognostic significance identified |
| Le et al | 2005 | EGF/HER-1 | 21 | IHC | 21 chemo-radiotherapy | 0 | Not reported | 100% | Not reported | High EGFR expression seen in all 21 specimens |
| Alvarez et al | 2006 | EGF/HER-1 | 38 | IHC and FISH | 38 chemo-radiotherapy | 0 | 22.6 | 55% | Not reported | No prognostic significance identified |
| Ajani et al | 2009 | EGF/HER-1 | 30 | IHC | 30 chemo-radiotherapy | 0 | Not reported | 87% | RR = 0.40 for DFS | No prognostic significance identified |

Abbreviations: DFS = disease-free survival; IHC = immunohistochemistry; FISH = fluorescent in situ hybridisation; RR = relative risk. *Patients included in final biomarker analysis. **In studies where patients have been differentiated by the presence or absence of tumour biomarker overexpression, overexpression is considered as biomarker positive.
mutationally inactivated in patients with SCC of the oesophagus and this genotype correlated with poorer survival rates (Sturm et al, 2001). The M30 protein is a monoclonal antibody that recognises a neoeptope of cytokeratin 18 produced during apoptosis, thus serving as a marker of spontaneous apoptosis (Carr, 2000).

Allal et al (2003) assessed the prognostic significance of apoptotic regulatory proteins in anal carcinoma. The proportion of tumours expressing Mcl-1 and Bcl-2 were 64 and 42%, respectively (Allal et al, 2003). Although Mcl-1 was found to have no prognostic significance, lack of Bcl-2 expression was an independent negative factor for local recurrence (RR 4.66, \( P = 0.0015 \)) and also for DFS (RR 4.11, \( P = 0.001 \)) (Allal et al, 2003). In the same study, Bax and M30 expression was 42 and 12%, respectively, but neither showed correlation with clinical outcome in the 98 patients studied (Allal et al, 2003).

Two other studies have investigated Bcl-2 in patients with anal cancer, but there was no significant association with survival (Le et al, 2005; Ajani et al, 2009).

Cyclins

Cyclins are a family of proteins that function in conjunction with CDKs to positively regulate specific cell cycle transition points (Bartek and Lukas, 2001). They have been studied extensively as regulators of cell cycle progression. The prognostic significance of three members of the cyclin family (A, D1 and E) has been studied in anal carcinoma (Table 5) (Allal et al, 2004; Le et al, 2005; Nilsson et al, 2006). All three cyclins govern cell cycle progression at the G1/S transition point, and cyclin A additionally exhibits regulatory control at the G2/M transition point (Pagano et al, 1992). Nilsson et al (2006) studied 215 patients with anal SCC and identified high cyclin A expression in 51% of these patients. This was significantly associated with better tumour-specific (81 vs 64%, \( P = 0.009 \)) and overall survival (77 vs 59%, \( P = 0.005 \)) when compared with tumours with low cyclin A expression (Nilsson et al, 2006). Furthermore, a lower rate of isolated locoregional failure was seen in patients with tumours expressing high levels of cyclin A (\( P < 0.05 \)) (Nilsson et al, 2006). Uni- and multi-variate analyses revealed that cyclin A was an independent predictive marker of survival (hazard ratio 0.54, 95% CI). In the two studies of cyclin D1 and one study of cyclin E in patients with anal cancer treated with radiotherapy + chemotherapy, pretreatment tumour levels of these proteins were found to have no association with patient outcome (Allal et al, 2004; Le et al, 2005).

| Authors                      | Year | Biomarker | Patients (n) | Method | Primary surgical treatment (n) | Mean (median) follow-up period in months | Biomarker +ve patients (%) | Survival (biomarker +ve vs -ve patients) | Correlation with prognosis       |
|------------------------------|------|-----------|-------------|--------|------------------------------|------------------------------------------|----------------------------|----------------------------------------|-----------------------------------|
| Nilsson et al 2006 | Cxyn A | 215       | IHC         | Not reported | 7 local excision, 6 APR | Not reported | 51% | 77 vs 59% 5-year overall survival | High cyclin A expression associated with improved overall (\( P = 0.005 \)) and DFS (\( P = 0.009 \)) | No prognostic significance identified |
| Allal et al 2004 | Cyclin D1 | 98      | IHC         | 47 radiotherapy alone | 51 chemoradiotherapy | 0 | (124) | 34% | 57 vs 67% 5-year DFS | No prognostic significance identified | No prognostic significance identified |
| Le et al 2005 | Cyclin D1 | 21      | IHC         | 21 chemoradiotherapy | 51 chemoradiotherapy | 0 | (124) | 33% | Not reported | No prognostic significance identified | No prognostic significance identified |
| Allal et al 2004 | Cyclin E | 98      | IHC         | 47 radiotherapy alone | 51 chemoradiotherapy | 0 | (124) | 33% | Not reported | No prognostic significance identified | No prognostic significance identified |

Abbreviations: APR = abdominoperineal resection; DFS = disease-free survival; IHC = immunohistochemistry. a Patients included in final biomarker analysis. In studies where patients have been differentiated by the presence or absence of tumour biomarker overexpression, overexpression is considered as biomarker positive.

Cyclin A is a nuclear antigen associated with the late G1, S and early G2 phases of the cell cycle (Shin et al, 1994). It has a vital role in DNA synthesis and initiation of cellular proliferation. A single study has assessed the prognostic significance of PCNA in anal carcinoma, in which immunohistochemistry was used to analyse tumour samples obtained from 62 patients (Table 6) (Grabenbauer et al, 1998). There was no correlation with clinical outcome, and there have been no novel data identifying the prognostic value of PCNA in anal SCC.

Proliferating cell nuclear antigen (PCNA) is a nuclear protein associated with the late G1, S and early G2 phases of the cell cycle (Shin et al, 1994). It has a vital role in DNA synthesis and initiation of cellular proliferation. A single study has assessed the prognostic significance of PCNA in anal carcinoma, in which immunohistochemistry was used to analyse tumour samples obtained from 62 patients (Table 6) (Grabenbauer et al, 1998). There was no correlation with clinical outcome, and there have been no novel data identifying the prognostic value of PCNA in anal SCC.

Non-metastatic protein 23 (Nm23) Studies of the nm23 protein both in vitro and in vivo have revealed a potential role in suppressing tumour metastasis through mechanisms that may be dependent on its nucleoside diphosphate kinase (NDPK) activity (MacDonald et al, 1995). Holm and Tanum (1996) studied nm23 expression using immunohistochemical analysis in 96 patients with anal carcinoma (Table 6). Of these specimens,
Table 6  Studies of markers of proliferation, invasion and metastasis in anal carcinoma

| Authors           | Year | Biomarker | Patients (n)* | Method | Radiotherapy ± chemo-therapy (n) | Primary Surgical treatment (n) | Mean (median) follow-up period in months | Biomarker +ve patients* | Survival (Biomarker +ve vs -ve patients) | Correlation with prognosis |
|-------------------|------|-----------|---------------|--------|---------------------------------|-----------------------------|------------------------------------------|------------------------|------------------------------------------|---------------------------|
| Afal et al        | 1998 | MIB1      | 55            | IHC    | 31 chemo-radiotherapy           | 0                           | (94)                                     | 41%                    | 59 vs 67% 5-year DFS                  | No prognostic significance identified |
| Grabenbauer et al | 1998 | MIB1      | 62            | IHC    | 62 chemo-radiotherapy           | 0                           | (52)                                     | 34%                    | 73 vs 50% 5-year DFS                  | Higher MIB1 index associated with better colostomy-free survival (P = 0.04) |
| Indinnimeo et al  | 2000a,b | Ki-67 | 31           | IHC    | 13 chemo-radiotherapy 7 radiotherapy followed by local excision | 5 local excision, 6 APR | 74.4                                     | 65%                    | Not reported                           | No prognostic significance identified |
| Ajani et al       | 2009 | Ki-67     | 30            | IHC    | 30 chemo-radiotherapy           | 0                           | Not reported                             | Not reported            | RR = 0.98 for DFS                      | Higher levels of Ki-67 associated with longer DFS (P = 0.005) |
| Grabenbauer et al | 1998 | PCNA      | 62            | IHC    | 62 chemo-radiotherapy           | 0                           | (52)                                     | 32%                    | 78 vs 45% 5-year DFS                  | No prognostic significance identified |
| Holm and Tanum    | 1996 | nm23      | 96            | IHC    | 96 chemo-radiotherapy           | 0                           | 60                                      | 79%                    | Not reported                           | Cytoplasmic nm23 expression associated with shorter survival (P = 0.03) |
| Indinnimeo et al  | 2000a,b | nm23 | 22           | IHC    | 9 chemo-radiotherapy 5 chemotherapy | 2 local excision, 6 APR | 63.6                                     | 27%                    | Not reported                           | No prognostic significance identified |
| Bruland et al     | 2008 | MCM7      | 55            | IHC    | 9 chemo-radiotherapy 46 chemo-radiotherapy alone | 0                           | 86.4                                    | Not reported            | Not reported                           | High MCM7 correlated with improved cancer-specific survival (P = 0.011) |
| Holm and Tanum    | 1996 | Cathepsin D | 96         | IHC    | 96 chemo-radiotherapy           | 0                           | 60                                      | 50%                    | Not reported                           | No prognostic significance identified |

Abbreviations: APR = abdominoperineal resection; DFS = disease-free survival; IHC = immunohistochemistry; PCNA = proliferating cell nuclear antigen; MCM7 = mini-chromosome maintenance protein 7; nm23 = non-metastatic protein 23; RR = relative risk. *Patients included in final biomarker analysis. **In studies where patients have been differentiated by the presence or absence of tumour biomarker overexpression, overexpression is considered as biomarker positive.

A single study assessed the prognostic significance of Cathepsin D in anal cancer, and although 50% of tumours were Cathepsin D positive, the investigators found no correlation with overall survival (P > 0.10) or with the clinicopathological parameters of tumour stage, tumour differentiation or lymph node involvement (Table 6) (Holm and Tanum, 1996).

**Angiogenesis**

*Vascular endothelial growth factor (VEGF)* Neovascularisation is key to tumour invasion and metastasis, and VEGF has an important role in this angiogenic process through interaction with its complimentary VEGF receptors (Backer et al, 2009). Anticancer therapies have been developed that target the VEGF receptor in order to improve tumour response rates. Two studies have investigated the prognostic significance of tumour VEGF levels in anal cancer patients treated with chemoradiotherapy, but neither study identified a correlation with patient survival (Table 7) (Wong et al, 1999; Ajani et al, 2009).

**Microvessel density (MVD) and cluster of differentiation 31 (CD31)** In recent years, a widely established method of determining tumour vascularity has been through calculating MVD, which involves identifying vascular hot spots within the tumours and subsequently counting the number of individual microvessels seen (Pathak et al, 2008). MVD can thus be considered as a semiquantitative marker of angiogenesis. A number of endothelial surface markers have been used to examine MVD in malignant tumours, and among the three studies that have assessed the prognostic significance of MVD in anal cancer, one study used factor VIII (Wong et al, 1999) and two studies used the CD31 molecule (Table 7) (Indinnimeo et al, 2001; Nilsson et al, 2006). All three studies, however, failed to demonstrate a putative role for MVD in predicting anal cancer prognosis (Wong et al, 1999; Indinnimeo et al, 2001; Nilsson et al, 2006).
The study by Indinnimeo et al (2001) did however report a positive correlation between CD31 score and depth of tumour invasion \((P < 0.005)\) in anal cancer, which supports the hypothesis that tumour growth occurs through angiogenesis-dependent mechanisms.

**Tumour markers**

**SCC antigen (SCCAg)** Serum concentrations of SCCAg, a polypeptide subunit of a protein originally identified in malignant human cervical tissue (Kato and Torigoe, 1977), is elevated in several tumour sites including the anal canal (Petrilli et al, 1988; Indinnimeo et al, 1997). Goldman et al (1993) examined the prognostic significance of this tumour marker and reported a clear association between SCCAg levels and survival (Table 8). Higher pretreatment serum SCCAg levels correlated with reduced tumour-free survival \((P < 0.00005)\) and overall survival \((P = 0.02)\) using multivariate analysis (Goldman et al, 1993). Another study of SCCAg in anal cancer found no prognostic value of pretreatment serum SCCAg, but did note that SCCAg levels were significantly elevated in patients who had relapsed following primary treatment (Fontana et al, 1991).

**Carcinoembryonic antigen (CEA)** CEA, a complex glycoprotein that has long been established as a useful adjunct in monitoring patients with colorectal cancer (Goldstein and Mitchell, 2005), was investigated over a decade ago in patients with anal SCC undergoing combined chemoradiotherapy (Table 8) (Tanum et al, 1992). Of the patients, 19% were found to have raised serum CEA levels before treatment and 17.5% of tumours stained positive for CEA, but no correlation with clinical outcome was observed (Tanum et al, 1992).

**Sonic hedgehog (SHH) signalling**

**SHH and Gli-1** Sonic hedgehog is a secreted glycoprotein belonging to the hedgehog family of proteins that are essential to organogenesis (Ruiz i Altaba et al, 2002). SHH triggers a complex signal transduction pathway, the cellular response to which is mediated by three Gli proteins (Gli-1, Gli-2 and Gli-3) (Ruiz i Altaba et al, 2002). Gli-1 is a transcription factor that serves as an important regulator of mammalian hedgehog signalling (Ruiz i Altaba et al, 2002). Hedgehog signalling has been associated with failure to respond to chemoradiotherapy and also with increased incidence of tumour regrowth following treatment.

Ajani et al (2009) investigated the prognostic role in patients with anal cancer treated with chemoradiotherapy. Immunohistochemistry was used to measure tumour SHH and Gli-1 expression. Overexpression of both markers was demonstrated through multivariate analysis to be independent predictors of shorter DFS \((P = 0.02\) and \(P = 0.02\), respectively; Table 8).
A single preliminary study suggested that NF-κB may be of prognostic value in anal cancer, but there have not been any other confirmatory studies (Ajani et al., 2009). Studies of NF-κB, most notably haematological malignancies, demonstrate that aberrant expression of this transcription factor is associated with tumour growth, progression and resistance to chemotherapeutic agents (Baud and Karin, 2009). Moreover, the use of the reversible 26S proteasome inhibitor bortezomib, which inhibits NF-κB activity, is an effective and approved agent in the treatment of multiple myeloma (Baud and Karin, 2009). However, NF-κB-targeted therapy is yet to become as widely established in other human malignancies.

Other apoptotic regulators that were shown to correlate with anal cancer prognosis in a single study were the Bcl-2 and M30 proteins (Allal et al., 2003). Two other studies of Bcl-2 failed to identify a correlation with clinical outcome (Le et al., 2005; Ajani et al., 2009). These studies had a relatively small patient sample size and therefore the true prognostic impact of the Bcl-2 and other apoptotic-regulatory molecules may need larger cohorts. In the study of apoptosis-regulating proteins by Allal et al. (2003), subgroup analysis revealed that patients with Bcl-2-positive/p53-negative anal tumours had significantly higher 5-year DFS compared with patients with tumours expressing all other combinations of these two proteins. Furthermore, results from phase III trials of oblimersen (Genasense), an antisense nucleotide that targets Bcl-2, in chronic lymphocytic leukaemia showed improved survival rates only when given in conjunction with conventional chemotherapy but not when used alone (O’Brien et al., 2007). This suggests that future therapeutic targets may need to be used in conjunction with cytotoxic agents or other targeted treatments.

Data from two of the four studies of Ki-67/MiB1 supported a predictive role in the prognosis of anal cancer patients treated with chemoradiotherapy (Grabenbauer et al., 1998; Ajani et al., 2009). In these studies, high Ki-67/MiB1 positivity correlated with improved survival that may be related to heightened responses to antiproliferative chemotherapeutic agents. This is highlighted by the fact that in the two studies where no prognostic significance was found, there were a cohort of patients who were not treated with chemotherapy but received radiotherapy (with or without local excision) (Allal et al., 1998; Indinnimeo et al., 2000b). The question therefore remains as to whether the potential prognostic value of Ki-67/MiB1 is confined to certain treatment regimens and whether Ki-67/MiB1 index are likely to require more aggressive treatment regimens.

The oncological significance of the VEGF molecule is evident from the established use bevacizumab (Avastin), a humanised anti-VEGF monoclonal antibody in the treatment of metastatic breast, colorectal, renal and non-small cell lung carcinoma (Goerner et al., 2010). The two studies of VEGF in anal cancer reported no association with patient outcome (Wong et al., 1999; Ajani et al., 2009). The prognostic significance of VEGF in anal carcinoma needs further evaluation.

Investigation of molecular markers such as p16, PCNA, MVD and the RB protein has demonstrated a prognostic utility in anal cancer. However, the degree to which these findings are limited by methodological factors is unclear. Other proteins such as M30, cyclin A, SCCAg, SHH and Gli-1 were demonstrated to be of predictive value in individual studies but further trials are required to reproduce these findings.

Other cancers related to HPV infection continue to increase in incidence. HPV-associated head and neck SCC is associated with HPV 16 similar to anogenital SCCs. In situ hybridisation studies have demonstrated that HPV status correlates (Kuo et al., 2008) with treatment response, progression-free survival and overall survival in oropharyngeal SCC (Fakhry et al., 2008; Ang et al., 2010). In this setting, much debate exists regarding the optimal detection technique and whether P-16 should be used as a surrogate marker.
Future strategies may involve the application of such hypotheses to anal cancer with the potential for vaccine therapy in established HPV-associated disease. The National Cancer Institute Phase II trial has completed accrual in studying the effectiveness of human papillomavirus vaccine (HPV-16 E-6 and E-7 peptides) therapy in treating patients who have advanced or recurrent cancer of the cervix, vagina, penis, anus, oesophagus, or head and neck (National Cancer Institute, 2009). We await the results of these and other studies.

There is a growing appreciation of the fact that all clinical trials of emerging targeted treatments must incorporate biomarker studies in order to minimise the possibility of failing to appreciate significant activity of the drug in a biological subset of a given cancer type and to maximise the chance of response in a given patient. This is the all the more essential in the case of anal carcinoma, which is an uncommon cancer type that will limit the patient numbers available for clinical trials. Certainly, there are targeted treatments that have been shown to have activity in other EGFR-expressing cancers that need assessment in this tumour type, and biomarkers that have been shown to be helpful in other cancer sites should be prioritised for assessment. Any biomarker studies and validation thereof should aim to comply with Biomarker Task Force recommendations in order to ensure that results can be assessed in terms of realistic clinical utility (Dancey et al., 2010).

CONCLUSION

Biomarkers in anal cancer may possess the ability to tailor treatment to individuals. Certain markers such as the EGFR protein are potential treatment targets in this disease. It remains to be seen if biomarkers of response to anti-EGFR therapies shown to be of use in other carcinoma types are applicable to anal carcinoma. Larger prospective studies of single or panels of multiple biomarkers are necessary with standardised methodology. Novel study designs are required and future prospects include the use of DNA microarray-based gene expression profiling that has already been shown to provide valuable prognostic information in patients with breast cancer (Munkacsi et al., 2010). Pharmacogenomics has the potential to make a major contribution to biomarker identification and future directions of therapy in anal cancer. Within its realm, single-nucleotide polymorphism (SNP) analysis serves as a promising avenue for future research studies. Although the quest for prognostic markers in anal cancer is currently in its early stages, expanding research into this field is driven by the prospect of unravelling molecular characteristics of these tumours that may ultimately prolong patient survival and reduce the health and economic burden of this disease epidemic.

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