Cancer of the uterine cervix: Integration of molecular evaluation into management strategy and the concept of biological staging

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Carcinoma of the uterine cervix is the most common malignancy affecting Indian women. The biological behaviour of invasive cervical cancer is not always predictable. Even when the lesion is localized to the cervix, 15–20% of the patients have recurrences. The evaluation of cervical precancer is even more complex and the current approach is to assess malignant potential based on histological and cytological criteria. However, lesions at any point in the spectrum of premalignancy have been associated with subsequent invasion, reflecting the limitations of histological grading for predicting the risk of progression. Biological markers are measures of cellular events associated with specific stages of carcinogenesis. This definition indicates that the risk of tumour progression and/or biological behaviour could correlate with the quantitative degree and pattern of biomarker expression. A number of such markers are now available for the evaluation of cervical lesions. Molecular, biological and histopathological investigations of preinvasive and invasive carcinoma of the uterine cervix have shown the role of human papillomavirus (HPV) infection in cervical carcinogenesis. Molecular analysis of HPV DNAs has also provided information on their genomic organization, protein function and transcriptional regulation. Studies on the expression of E6 and E7 transforming proteins of certain high-risk HPVs have shown that these viruses play a role in carcinogenic progression by forming complexes with products of the tumor suppressor genes, *Rb* and *p53*. Studies have also shown the association of the oncogenes, *ras* and *myc* with HPV and cervical carcinoma. The role of HPV infection, E6–E7 transforming proteins, oncogenes and tumour suppressor genes in cervical carcinogenesis are discussed in this paper. Evaluation of these molecular markers can thus be used to elaborate the existing grading system for cervical lesions and could play a vital role in the management of cervical precancer and cancer.

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Cancer of the uterine cervix: a formidable problem

Carcinoma of the uterine cervix is the most common malignancy affecting the Indian women, well confirmed by the age-adjusted incidence rates reported by the population-based registries at Bombay, Delhi, Madras and Bangalore[1]. Hospital statistics also show the disease to account for about 12.3% of all cancers and 23% of all female cancers recorded at the Regional Cancer Centre, Thiruvananthapuram[2]. A similar picture exists in other southeastern countries, where cervical cancer has been reported to range from 25–45% of all cancers[3]. In the United States, it ranks seventh in frequency among women and eighth as a cause of death among women[4]. With nearly half a million women developing the disease annually, this malignancy continues to pose an unresolved health problem[4].

Premalignant processes in the uterine cervix and its evaluation

One of the most intensively studied issues in gynaecological pathology over the past 30 years has been the factor(s) involved in the progression of precancerous processes to invasive cancer. This question is of vital importance since it addresses the biological characteristics that typify neoplasia, the histological features that can be recognized as signifying 'high risk' and the possibility that the use of such information will simplify the management of women with abnormal Papanicolaou smears.

The normal approach in evaluating various stages of cervical carcinogenesis involves applying standard histological criteria to identify cervical intraepithelial neoplasia (CIN). Relative risk is more or less conveyed by grading of the lesion. Lesions termed CIN I, II and III correspond to the presence of mild, moderate and severe dysplasia/carcinoma *in situ*, respectively. A newer classification called the 'Bethesda System' was also recently introduced for reporting cervical lesions.

This classification includes a two-tiered system, 'low-grade' and 'high-grade' squamous intraepithelial lesions (SILs). Low-grade SILs correspond to CIN I while high-grade SILs correspond to CIN II and III lesions. It is presumed that lesions at the higher end of all classification spectrums possess a greater risk of progression to cancer if left untreated[5,6]. Nevertheless, lesions at any point in the spectrum have been associated with subsequent invasion, reflecting the limitations of histological grading for predicting the risk of progression[2,6]. It is, therefore, clear that routine histology and/or cytology may be inadequate to predict the precise process of cervical carcinogenesis.

Prognostic features in cervical cancer

The biological behaviour of invasive cervical cancer is not always predictable. Even when the lesion is localized to the cervix, 15–20% of the patients have recurrences[7,8]. Clinical prognostic factors include lymph node involvement, tumour size, histological type and grade of differentiation[9]. Lymph node involvement appears to be one of the most important factors, not only in patients with early stages of cervical cancer, but also those having para-aortic and multiple lymph node metastases are likely to develop distant metastases[7,8]. However, in many cases with involved lymph nodes there are no recurrences and conversely the absence of positive nodes does not ensure disease-free status. The problem, therefore, for oncologists is to try and predict accurately those tumours which will relapse in order to modify or modulate treatment protocol. It is thus important to establish biological markers which can be associated with disease course.

Biological markers and malignancy

Until recently, the scope of scientific study of biologic processes involved in early cancer development in humans was largely confined to the histological characterization of premalignancy[10]. Oral leukoplakia, cervical dysplasia and colonic polyps have served as useful markers of increased cancer risk in the involved tissues based on clinical appearance and histopathological examination. This, however, is extremely subjective and has considerable difficulty in determining which alterations are important predictors of future behaviour and which are coincidental. With the clarification of the carcinogenesis process, well-defined stages of initiation, promotion, conversion and progression have been identified[11]. This theory postulates specific events to occur at each step. Ideally, therefore, measurements of carcinogenesis can be achieved by studying biological markers associated with each intermediate step, giving rise to the concept of biological or intermediate markers[11].

Biological markers can be defined as measurable markers of cellular events associated with specific stages of the multi-step evolution and progression of carcinogenesis[12,13]. This definition indicates that the risk of carcinogenic transformation correlates with the quantitative degree and pattern of biomarker expression. Markers are specific, novel or structurally related cellular macromolecules, or temporally, spatially or quantitatively altered normal molecules associated with malignant (and in some cases with benign) cells. Thus, biological markers represent signals in a continuum of events between the start of carcinogenesis and the final expression of clinically evident disease[11–13].

The need for biological staging

The optimum staging system of malignant neoplasms describes the extent of the particular cancer and relates to its natural course. This classification should be uniform and should facilitate the exchange of information, be useful in the evaluation of the results of treatment, be able to forecast an outcome and give a sense of prognosis. Three significant events in the history of cancer determined by clinical examination before therapy is begun give the anatomic extent of the disease. These are tumour growth at the primary site (T), spread to regional lymph nodes (N) and distant metastases (M). The description of the extent of cancer or morphological evaluation and accurate histological evaluation are essential elements in a meaningful documentation of a tumour and should be used with consistency.

However, it is widely held that the current staging system for cervical cancer may have limitations. For example, the group staging is ambiguous because it includes cancers of different natural behaviours. Two-dimensional definitions that utilize only the size and location of cancer do not necessarily predict its metastatic potential. Certainly, local spread and distant metastases may have occurred long before they are evident by clinical examination. A similar problem is also evident with cervical precancers. As outlined earlier, lesions at any point in the premalignant spectrum have been associated with subsequent invasion, reflecting the limitations of histological grading for predicting the risk of progression[6].

It is with these limitations in mind that the American Joint Committee on Cancer have in their recent publication[14] stated that *in the future biologic markers and other parameters may have to be added to those of anatomic extent in classifying cancer.* A number of prognostic indices have been published for various cancer sites[14–18]. Although subject to a few drawbacks, these markers have depended on small numbers of cases. In addition, not all components of the AJCC/UICC categories were considered although most prognostic

indicators such as tumour size and nodal status were included. However, they still represent efforts to organize logically our knowledge of prognostic indicators for cancer patient management.

Basic research has produced new markers which have the ability to estimate disease progression and prognosis. These include amplified oncogenes, oncoproteins, paracrine and autocrine growth factors, extent of aneuploidy, cell receptors and proliferation markers. Such prognostic markers will probably not apply to all stage groupings. Their important contribution will be for patients with early-stage disease. In addition, prognostic markers will perform new roles for early cancer detection, i. e. throw new light on the process of carcinogenesis. They will not only serve as intermediate endpoints for screening but will also clarify the contribution of lead time and length bias by identifying tumours that are slow-growing. It is, therefore, obvious from these points that biological markers can play a vital role in the management of both cervical precancer and invasive cancer.

Molecular markers for cervical lesions

The development and biological behaviour of preinvasive and invasive lesions of the uterine cervix is associated with the expression of a number of molecular markers. Such prognostic markers may improve our ability to estimate the disease outcome, especially for patients with early-stage disease and those with precancerous lesions. Among the best-studied molecular markers of cervical cancer and precancer are human papillomavirus (HPV) infection, HPV transforming proteins – E6 and E7, the oncogenes *ras* and *c-myc* and tumour suppressor genes *Rb* and *p53*.

Human papillomaviruses and cervical cancer

Molecular, biological and histopathological investigations of preinvasive and invasive squamous cell carcinoma of cervix performed over the last decade have demonstrated the presence of human papillomavirus (HPV) nucleotides in 80–90% of precancerous and cancerous lesions[3,19]. There are approximately 65 different subtypes of HPVs which have been reported[20]; and, of these, at least 20 have been found to be associated with the female genital tract. These HPVs can be further classified as either 'high-risk' or 'low-risk' based on whether or not the genital tract lesions with which these HPVs are associated have a risk for malignant progression. The low-risk viruses include HPV-6 and HPV-11, associated with benign genital warts and low grades of CIN, whereas 'high-risk' viruses such as HPV-16 and HPV-18 are associated with the majority of cervical carcinoma and high-grade dysplastic lesions[20-22].

Distribution and behaviour of HPVs in the uterine cervix

Several histologic and molecular studies have demonstrated a differential distribution of HPVs in cervical cancer and in genital tract lesions[23,24]. HPV-6 and 11 are usually associated with lesions that are low-grade CIN lesions. Although HPV-16 and 18 have both been evaluated as high-risk viruses, there is some evidence that they behave differently[23,24]. The frequency of HPV-18 in low-grade CIN is disproportionately low compared to its frequency in invasive cancers. Adenocarcinoma of the cervix also appears to be associated with HPV-18 in up to 55% of cases[25]. HPV type 16 DNA sequences have been detected in considerable frequency in normal cervical epithelium, showing that HPV virions can exist in apparently normal tissues[26,27]. It is also suggested that in occult infection, the virus infects a large number of cells but is present in a low copy number[27].

The prevalence of HPV-6 and 11 in benign and low-grade lesions suggests that a significant proportion of CIN I lesions should not only be regarded as part of a pathologic continuum to cervical cancer but should also represent a specific manifestation of HPV-6/11 infection. CIN I lesions tend to either regress or persist but rarely progress. High-grade lesions are often found to have a higher rate of basal cells proliferation and are more likely to have other genetic alterations that can result in the establishment of a malignant phenotype[28]. The prevalence of HPV type 16 and 18 in invasive tumours suggests a particular carcinogenic potential of these virus subtypes[29,30]. A number of current studies have indicated that the presence of HPV-16 and 18 appears to be a major risk factor for progression of precancer to carcinoma, thereby emphasizing the clinical value of their identification[31].

Although HPV-16 and 18 have been detected in approximately 70% of invasive carcinomas, HPV-18 displays certain unique characteristics suggesting that it operates differently from HPV-16. It has been proposed that the deficit of HPV-18 in CIN compared to invasive carcinoma may reflect rapid transit time through the CIN or that HPV-18-related lesions may short-circuit the usual pathway and progress to invasive carcinoma directly from low-grade lesions[28]. It has been suggested, therefore, that HPV-18 may account for what has been termed 'rapidly progressive cervical carcinoma'[28]. Such patients are usually young and present with more advanced disease than is initially suspected. The tumours tend to be poorly differentiated and pursue a highly aggressive course. Studies comparing clinical and pathological features of patients with cervical cancers containing HPV-18 have shown that these tumours have a higher frequency of pelvic lymph node metastasis[32]. The recurrence rate was 45% in patients with HPV-18 tumours compared to 16% for HPV-16-containing

tumours[33]. *HPV-18 also shows a tendency to be* preferentially distributed in adenocarcinomas[25] and in highly aggressive small-cell undifferentiated carcinomas[34].

Follow-up studies of intraepithelial lesions suggest that those containing HPV-16 and 18 have a higher propensity to progress than do lesions containing HPV types 6 and 11 (ref. 35). However, a long-term prospective study reports that the low-risk HPV types were found in carcinoma *in situ* (CIS) lesions as frequently as the high-risk types, HPV-16 and 18 (ref. 36). This, thus, suggests a possible similarity in the biological behaviour of these HPV groups and that infection by HPV-6 and 11 by no means excludes the possibility for progression into invasive cancer.

Interaction of viral and cellular genes

The life cycle of HPV is tightly linked to squamous cell differentiation[31,35]. Following infection, the virus either *remains dormant (the latent stage) or undergoes active* replication, resulting in the synthesis of complete and infectious virus particles. It is thought that HPV infects the basal layer of the epithelium and replicates extrachromosomally as an episome[19,21]. Under certain circumstances, the viral genome may become integrated into the cellular genome. Integration disrupts the viral genome in the E1–E2 regions (regions that code for nonstructural proteins), resulting in the failure of transcription of the late genes (regions that code for structural proteins) and possibly in uncontrolled transcription of the E6 and E7 genes[31,37]. The E6–E7 regions code for proteins that are involved in the regulation of viral growth[31,37].

Expression of E6/E7 transforming proteins of HPV

Morphologically, HPV infection in low-grade SILs is determined on the basis of koilocytosis in the superficial layers of the thickened epithelium. Molecular analysis of recombinant HPV DNAs has provided information on their genomic organization, protein functions and transcriptional regulation. Several lines of evidence suggest the possible mechanism of HPV-induced carcinogenesis.

Studies on cervical cancers and cell lines derived from cervical cancers that are HPV-positive have demonstrated active transcription and translation of the E6–E7 regions of high-risk virus types HPV-16 and HPV-18 (refs 37, 38). The E6 and E7 regions of the high-risk viruses are able to immortalize primary human keratinocytes[39–41]. Persistent E6 and E7 expression is essential in maintaining the transformed phenotype[41]. The demonstration of the transforming properties of the E6 and E7 oncoproteins of the high-risk HPVs supports a role of *these viruses in carcinogenic progression.* The HPV genomes in cervical cancers and in derived cell lines are transcriptionally active and patterns of viral RNA expression are specific with regular expression of E6 and E7 genes[42]. It has been proposed that the integration of the viral genome in cervical cancers provides a selective advantage leading to uncontrolled proliferation of the cell due to the deregulated expression of the E6 and E7 genes[21].

Expression of oncogenes

Cellular oncogenes are activated by overexpression, transposition or structural alterations within the gene sequences and is assumed to be associated with neoplastic growth of human tumours[43]. Of the oncogenes, *myc* and *ras* have been found to be commonly expressed in cervical cancer[44]. The overexpression of the *c-myc* gene has been observed in cervical carcinomas[45–54]. Studies of squamous cell carcinomas of the uterine cervix have shown c-myc overexpression in 44% of stage I and II tumours[47,54]. Studies have also shown that activation of the *c-myc* gene is associated with a poorer prognosis[46]. In a study of 72 patients with stage I or II tumours, Riou *et al.*[48] found high levels of *c-myc* RNA in 25 samples. On estimating a univariate risk of relapse, it was found to be associated with c-myc overexpression, nodal status and geographical origin (study patients were from Africa and France). A subsequent multivariate analysis showed only *c-myc* overexpression and nodal status to be associated with risk of relapse. Of the 25 patients with high levels of *c-myc* RNA, 13 relapsed. By contrast, of the 47 patients without c-myc overexpression, only 4 relapsed. Thus, a significant association between raised levels of c-myc transcripts and risk of relapse was found irrespective of other prognostic factors[48]. Studies also show that early-stage invasive cervical cancers with *c-myc* gene overexpression and those containing no detectable HPV DNA sequences were associated with a high risk of relapse[47,49,50]. A recent study has shown that c-myc overexpression and HPV negative status are two independent, prognostic indicators strongly related to the risk of distant metastasis[51]. Studies have also shown that the *c-myc* gene is more frequently overexpressed in grade III CIN than in grade I CIN[52] and that HPV sequences are preferentially integrated near *myc* genes in invasive genital cancers[53]. It is thus suggested that overexpression of the *c-myc* gene might play an important role in the oncogenesis of cervical carcinoma and that amplification is not always necessary for the activation of this gene[54].

There are also some reports on the association of the *ras* oncogene with cervical carcinoma. A 3–30-fold amplification of *c-Ha-ras* gene has been reported in advanced cervical carcinoma[55]. Activation of *c-myc*

gene was found in tumours with mutation or deletion in the *c-Ha-ras* gene[55]. To examine the correlation between *ras* gene expression and the development of cervical cancer, Sagae *et al.*[56] studied the reactivity of cervical intraepithelial neoplasia and microinvasive lesions of the cervix by using an anti-ras p21 monoclonal antibody, rp35. Their data showed that the frequency of p21 increased with higher grades of dysplasia, suggesting that p21 positivity increases during early carcinogenesis from CIN to invasive carcinoma. The same authors have also reported that the expression of ras p21 is a prognostic indicator for cervical cancers but the mode of its prognostic correlation was dependent on tumour histology[57]. Another finding of clinical significance was a report showing a correlation between elevation of p21 expression in cervical carcinomas and the incidence of lymph node metastases[58].

Expression of tumour suppressor genes

In vitro studies suggest that HPVs interact with cellular genes that normally regulate cellular proliferation. The E6 and E7 proteins of high-risk HPVs have been shown to form complexes with proteins encoded by the tumour suppressor genes, *p53* and retinoblastoma susceptibility (*Rb*) gene, respectively[59,60]. The demonstration of an interaction between the E7 gene product and the Rb protein suggests that the formation of such a complex might inactivate the Rb gene, resulting in loss of cellular growth control in a manner similar to that seen in cells homozygous for retinoblastoma gene deletion[61]. The Rb gene product is thought to play a key role in regulating intracellular signalling pathways[61]. The E7 proteins from low-risk HPVs (types 6 and 11) associated with the Rb protein have approximately tenfold lower affinity than do the E7 proteins of the high-risk HPV types 16 and 18. This differential binding may be partially responsible for the clinical differences between low- and high-risk HPVs. Additionally, the binding site of E7 on Rb protein has also been shown to bind to the product of *c-myc* and *N-myc* oncogenes[62]. The binding of E6 to p53 protein promotes degradation of the latter[63]. This selective degradation of p53 by the E6 protein is an important mode of action of this dominantly acting oncoprotein.

These findings give rise to a possibly new evaluation scheme to grade the progression of cervical precancer.

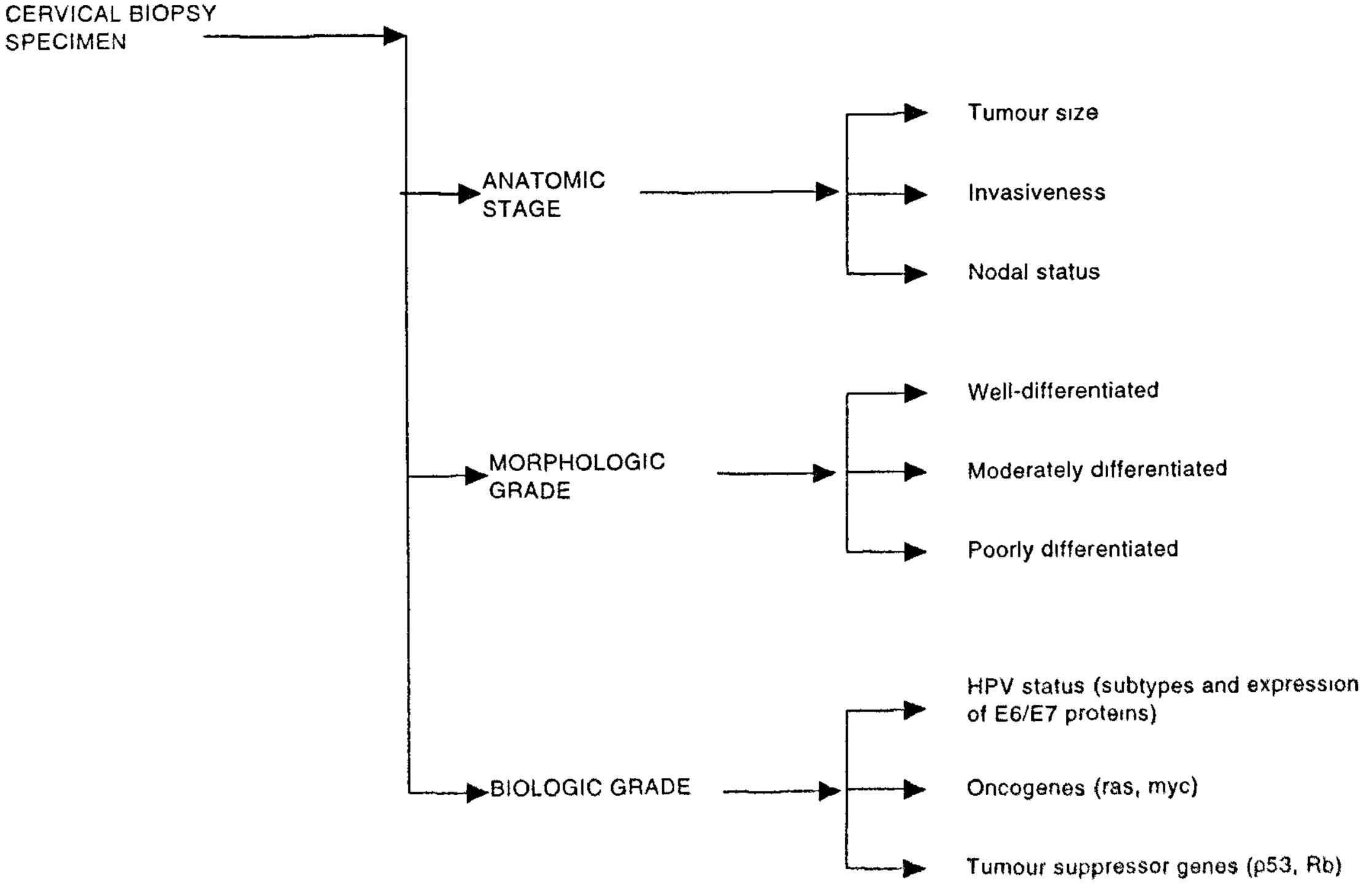

Figure 1. The three-tier AMB (anatomic–morphologic–biologic) evaluation protocol for cervical carcinoma

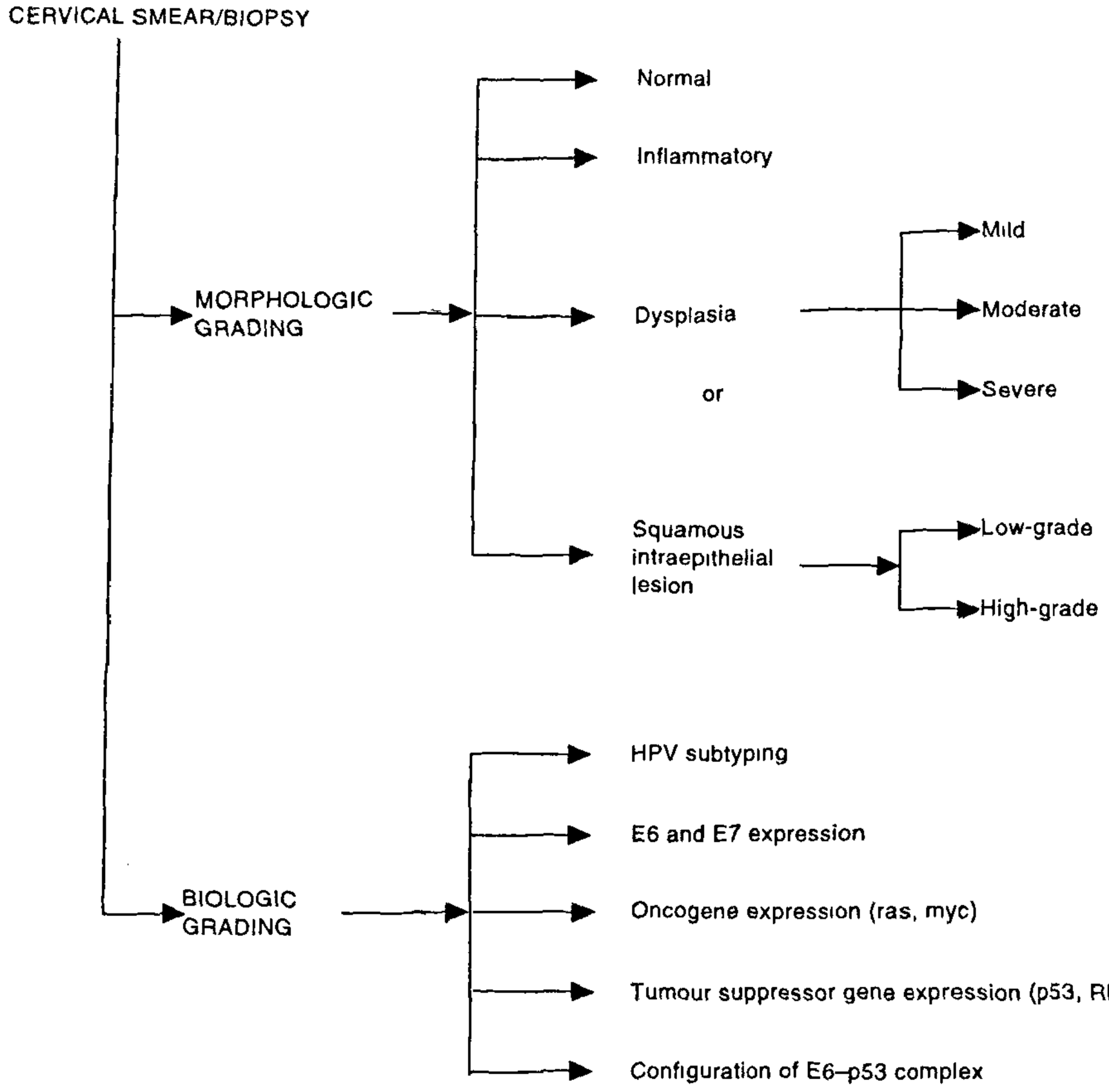

Figure 2. The two-tier MB (morphologic–biologic) evaluation protocol for cervical precancer (*Note* Many laboratories still continue to use the Papanicoloau grading while more recent guidelines suggest the use of the Bethesda system (SIL classification) The prognostic implications of both systems in relation to biological classification has to await the results from experimental studies Hence, both options have been shown)

Thus, HPV infection defined as the presence of HPV DNA or HPV DNA and E6 protein expression may not be sufficient for tumour progression. The argument is that the presence of DNA alone may not be enough to cause active infection because it could be episomal or otherwise non-productive. Protein expression would be a necessary requirement for HPV activity. However, protein expression, while a necessary step, may also not be sufficient as it may need to be complexed with p53 (Brian Herman, University of North Carolina, personal communication). The latter aspect has been demonstrated elegantly in cervical carcinoma cell lines recently[64]. Using high spatial resolution imaging by confocal microscopy with double fluorescence staining, p53 and E6 were shown to have similar cytoplasmic distribution, implying that these two proteins may exist as a cytoplasmic complex. To further substantiate this implication, these authors provided direct evidence of a close association between p53 and E6 within individual cells using fluorescence resonance energy transfer microscopy[64]. All these results, therefore, support the contention that inactivation of p53 by complex formation with E6 may be a critical step in the malignant transformation.

Studies on p53 mutations in HPV-positive and HPV-negative cervical cancer cell lines have reported the absence of p53 mutations in HPV-positive samples, while HPV-negative samples showed the presence of p53 mutations[65–67]. These results suggest that HPV-associated cervical carcinomas will have the wild-type p53 gene, whereas tumours without HPV will have mutant p53. The mutations in p53 were located in highly

conserved regions and resulted in aberrant proteins that were not phosphorylated and were unable to complex with the viral oncoprotein[65]. It has also been suggested that in HPV positive cervical lesions, p53 inactivation occurs via the known mechanism of association between HPV E6 and p53 proteins, whereas in most other tumours p53 function is altered by changes in amino acid sequence[66]. Thus p53 gene mutations and HPV-mediated functional p53 inactivation serve the same goal. However, the clinical significance of the two pathways differ. HPV negative cervical tumours often have a poorer prognosis than HPV-positive ones[68]. This, therefore, indicates that the different roles of p53 during the carcinogenic process (inactivation in HPV-positive tumors and mutation in HPV-negative tumours) have a prognostic significance. Yet another significant event is the level of p53 gene expression. A recent study provided interesting data of clinical significance[69]. Levels of p53 mRNA were not found significantly different in HPV-negative and positive tumours. However, low mRNA levels were observed in advanced stages of the disease (stages III and IV). The authors, therefore, suggested low transcript levels of p53 to be associated with tumour aggressiveness. Alternatively, it may also be an evidence suggesting p53 to be involved in the early phases of cervical tumour growth.

Conclusions

The optimum staging system for malignant neoplasms should also relate to the natural history of the disease besides describing the extent of the disease. It should also be uniform, should facilitate the exchange of information, be useful in the evaluation of treatment results, be able to forecast an outcome and give a sense of prognosis. It is widely held that the current clinical staging and histological staging for cervical cancer may have limitations[44,70]. For example, group staging is ambiguous because it includes cancers of different natural behaviour. Two-dimensional definitions that utilize only the size and location of cancer do not necessarily predict its metastatic potential. Certainly, local spread and distant metastasis may have occurred long before they are discernable by clinical examination. The clinical significance of histologic grading of cervical carcinoma is controversial[70]. Most authors use the modified three-grade system first suggested by Broders or modifications of Wentz–Reagan classification[70]. Both these grading systems do not predict any clinical outcome[70]. A similar, if not more important, problem is also evident with cervical precancers. Lesions at any point in the premalignant spectrum have been associated with subsequent invasion, reflecting the limitations of histologic grading for predicting the risk of malignant progression. As described earlier, it is with these limitations in mind that the American Joint Committee on cancer have called for a biological staging system to be integrated with the existing clinical and pathological grading systems[70]. Research has revealed new markers that may improve our ability to estimate disease outcome. The development of intraepithelial neoplasia and biological behaviour of invasive cervical cancer has been associated with a number of molecular markers. Expression of these markers can be evaluated using refined techniques such as immunohistochemistry, *in situ* hybridization, southern blot and polymerase chain reaction[11]. Such prognostic markers will probably not apply to all stage groupings and their most important contribution will be for patients with early-stage disease. In addition, molecular evaluation will perform new roles for early cancer detection[71]. We have hypothesized two such evaluation systems, one for cervical precancer and the other for invasive carcinoma (Figures 1 and 2). Though such recommendations may seem ambitious, we believe that the time is now ripe to elaborate and supplement the existing staging system. Molecular evaluation will thus have a vital role in the management of both cervical precancer and invasive cancer.

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