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Special Paper

Quality Control of Immunohistochemical Evaluation of Tumour-associated Plasminogen Activators and Related Components

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The plasminogen activation (PA) system is involved in the breakdown and remodelling of the extracellular matrix. In the case of cancer, this is a prerequisite for invasion and metastasis. The expression of urokinase-type plasminogen activator and plasminogen activator inhibitor type 1 in particular have been reported to be of clinical and prognostic value. This has primarily been proven in the case of breast carcinoma and colon carcinoma, using the enzyme-linked immunosorbent assay (ELISA) as a quantitative assay to determine the level of expression. Immunohistochemistry is another technique to investigate the presence of PA components. It allows assessment in a semiquantitative way and informs in addition on the specific distribution within the tissue. To take full advantage of the benefits of immunohistochemistry, it is important to aim at optimal quality in all steps influencing the final judgement of the staining results. These various steps are highlighted and discussed in this paper.

Key words: immunohistochemistry, quality control, plasminogen activation, technique
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INTRODUCTION

THE INTEREST in urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), their inhibitors plasminogen activator inhibitor type 1 (PAI-1) and type 2 (PAI-2) and the uPA-receptor (uPAR) in solid tumours has greatly increased since it has been shown that the presence of some of these components in malignant tumour lesions correlates with an unfavourable prognosis in terms of shorter disease-free interval and shorter survival. Duffy and colleagues [1, 2] and Jänicke and associates [3] demonstrated that increased levels of uPA in breast cancer tissue samples were associated with an unfavourable outcome. These early findings were confirmed by extension to larger series of patients [4–6]. Currently, it is generally accepted that not only uPA but also its inhibitor PAI-1 are strong parameters for unfavourable prognosis in node negative breast cancer patients, independent of tumour size or steroid hormone receptor status [4, 6–8]. The components tPA, PAI-2 and uPAR have also been described in relation to prognosis, indicating that tPA and PAI-2 are favourably, whereas uPAR is unfavourably, related to prognosis [9–11]. In addition to breast cancer, components of the plasminogen activation (PA) system also have a prognostic value in colorectal, gastric, lung, bladder and ovarian cancer [12, 13]. Also, other proteases were reported to have prognostic implications in breast cancer, such as cathepsins B, D and L [14, 15]. All of these studies were performed by enzyme-linked immunosorbent assay (ELISA) on freshly frozen primary tumour tissue, for which 100–300 μg of representative tumour tissue is needed. Other types of proteases (e.g. metalloproteases) are currently under investigation in order to define their impact on prognosis. All data indicate the important role of proteases in the assessment of prognosis in various solid cancers. For further

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information on these proteases and related components a summary is given in Table 1.

The ELISA approach gives an excellent impression on the amount of proteases or related components present in tumour tissue. However, no information is given on their cellular distribution. This distribution is scientifically interesting and may be of clinical relevance, as both tumour cells and/or stromal cells may produce these proteins, and because tumours may show considerable heterogeneity of expression. For future intervention studies using drugs that may interfere with proteases and/or related components, it may be of importance to know their exact cellular distribution. In order to obtain this information, immunohistochemistry is the method of choice, as it shows the cellular distribution of proteins in cell preparations and in tissue sections. Furthermore, the extent of the expression estimated by immunohistochemistry may also give prognostic information.

Immunohistochemistry is widely used in diagnostic histopathology and requires the interpretation of an expert pathologist. In principle, the assessment can be performed on small tissue samples, both on frozen sections and sections of formaldehyde-fixed, paraffin-embedded tissue material. As biopsies and excised tumour specimens get smaller as a consequence of earlier diagnosis, it is important that immunohistochemical studies can be executed on the material routinely processed for conventional histopathology. Within the framework of the BIOMED-1 programme of the European Union, a Concerted Action was established to determine the clinical relevance of proteases in tumour invasion and metastasis. It was decided to aim for those proteases serving as prognostic parameters that could be detected on a routine basis. This goal, however, requires a strict quality control system, both for ELISA and immunohistochemistry, Because of its different specifications, the quality assurance regarding ELISA for proteases of the PA system and related components has been described elsewhere [16, 17]. In the present contribution, a protocol describing a quality control system for immunohistochemical evaluation is discussed, based on several consensus meetings and laboratory practice of the BIOMED-1 project group members. The system is Europe wide, as it was set up in close collaboration with members of the EORTC Biomarker Study Group and the EORTC Melanoma Cooperative Group.

DESCRIPTION OF A QUALITY CONTROL SYSTEM IN IMMUNOHISTOCHEMISTRY

A quality control system is defined in terms of good laboratory practice as the organisatorial structure, responsibilities, procedures, processes and facilities in connection with quality assurance. Quality comprises three aspects: managerial, technical and interpretative (medical and/or scientific). Managerial or system quality assurance includes an established network of well-equipped (expert) laboratories with access to clinical information and data management and analysis. The network should be interlinked by an adequate data storage and information system. It should have a permanent character in order to guarantee continuity of the activities, reporting of the results and a forum of openly interacting specialists. For these reasons, an EORTC Cooperative Group or Study Group is ideally suited for these purposes. A European BIOMED-1 Concerted Action can be regarded as a transiently subsidised transnational task force on a certain topic of limited duration, that can help to solve important issues and implement the results into the ongoing regular activities.

Technical quality comprises the reagents and methods used, including proper controls. For general use they should be standardised as much as possible, in order to decrease technical variation and misinterpretation of data. Furthermore, reagents should be of consistent quality, if possible of unlimited supply, exchanged between laboratories on a regular basis and commercially available. In immunohistochemistry, one has to keep in mind that the quality of the interpretation is markedly influenced by the relative complexity of the expression patterns and the specific expertise of the trained observer. Standardisation should be accomplished by using a reliable and simplified scoring system that assures a high level of accuracy and a high level of reproducibility.

Table 1. *Extracellular proteolytic systems involved in invasive growth of tumour cells*

Type	Components	Features
Matrix metalloproteases (MMPS)		Zn^{++}-dependent
	Collagenases	Cleave collagen types I, II and III
	Gelatinases	Cleave collagen type IV and fibronectin
	Stromelysines	Cleave various extracellular matrix components
	Inhibitors (TIMP-1 and -2)	Inhibit activated MMPs
Serine proteases		
	Plasmin	Cleaves fibrin and extracellular matrix components (laminin, protein core of proteoglycans, collagen type IV)
	Plasminogen activators: Tissue-type (tPA) and urokinase-type (uPA) with corresponding receptor uPAR	Convert plasminogen into active plasmin Activate collagenases
	Inhibitors (PAI-1 and PAI-2)	Inhibit enzymatically active uPA and tPA
Aspartyl protease		
	Cathepsin D	Cleaves various extracellular matrix components
Cysteine proteases		
	Cathepsins B and L	Cleave various extracellular matrix components

Preferably the scoring system should lead to semiquantitative data that can be analysed properly using statistical computation. The degree of reproducibility is tested on a routine basis by inter- and intra-observer studies. Such systems including the aspect of 'Result evaluation of the Professional' are termed Integral Quality Control Systems. More details on the quality of the interpretation of the expression of proteolytic factors in tumour lesions are given below.

TECHNICAL QUALITY OF IMMUNOHISTOCHEMICAL DETECTION OF PLASMINOGEN ACTIVATORS AND RELATED COMPONENTS

Important aspects of the technical quality include the type of reference material and antibodies selected, the method of tissue processing, the most suitable immunohistochemical staining procedures and the approaches for antigen retrieval (Table 2). As reference material, it would be ideal to use rat xenografts of cell lines with well-characterised expressions of the components of interest, since murine antibodies can also be tested in this system. In our studies on immunohistochemistry for components of the PA system [18] we used the human melanoma cell lines BLM, MV3, 530 and IF6 which have earlier been characterised with regard to expression of these components [19]. MV3 and BLM were shown by Northern blotting and ELISA to be markedly positive for uPA and PAI-1, whereas the levels in 530 and IF6 were below the detection limit. Bianchi and colleagues [20] characterised the human breast carcinoma cell lines BT474 and MB231, and found that the BT474 is negative for uPAR, whereas MB 231 expresses considerable levels of uPAR-specific mRNA. The human melanoma cell line M24met is also found to contain high levels of uPAR [21]. In case only a non-tumorigenic cell line is available, processing a cell pellet for histological sections can be considered. It is advised not to merely use the very high expresser controls but a graded series of positives as well as a negative in a panel for antibody testing.

Based on the properties mentioned in Table 2, a series of monoclonal and polyclonal antibodies were collected for detecting uPA, tPA, PAI-1, PAI-2 and uPAR expression on paraffin sections [18]. Polyclonal antibodies should be used only if an ample supply can be guaranteed. Before employing a new batch of polyclonal antibody the staining results should be carefully compared with those of the previous one(s). In addition, Western blotting on tumour cytosols would be prudent as different batches may show differences in specificity. Before testing large series of human tumour lesions, antibodies—both polyclonal and monoclonal–should be analysed on tissue controls with a known expression, as established by ELISA. Furthermore, staining patterns on frozen and on corresponding paraffin sections from the same lesions should be compared. Based on these explorative staining procedures, the proper conditions can be established, including the dilution of the first and second antibodies. As fresh tissue samples are taken, both deep-frozen and formaldehyde-fixed, paraffin-embedded material should be assessed. Standardised fixation by buffered formaldehyde is very important for an adequate balance between accessibility of antigenic determinants and good preservation of morphology. It is strongly recommended to use phosphate-buffered formaldehyde (pH 7.4) of high quality only, and to fixate the tissues for a maximum duration of 24 h at room temperature. Unbuffered formaldehyde is associated with a lowering of the pH influencing antigenicity of target molecules. The optimal fixation time depends on the tissue volume. For core biopsies, adequate fixation may occur within only a few hours. Larger specimens, however, may need 12 h. A shorter fixation time may lead to suboptimal morphology, whereas immune staining may not be very distinct due to poor fixation. On the other hand, prolonged fixation may result in decreased immune reactivity due to extended cross-linking of antigens [22–24]. It has not been settled so far whether frozen sections should be postfixed in acetone, methanol or formaldehyde.

A large experience exists in the participating laboratories on the use of different immunohistochemical staining procedures. In several diagnostic histopathological laboratories, an indirect avidin–biotin (AB) procedure is routinely used.

Staining of paraffin sections may be improved by application of antigen retrieval procedures. These may include harsh treatments, e.g. digestion with trypsin or pronase. Furthermore, precooking of sections in a microwave oven or boiling

Table 2. Technical aspects of quality assessment in immunohistochemistry

Reference material
Nude rat xenografts of human tumour cell lines with and without expression of the components to be determined, as measured by enzyme-linked immunosorbent assay (ELISA)
Fresh human control tissues positive for the various components
Fresh tumour lesions should be deep-frozen. Part of the lesion should be fixed overnight using buffered formaldehyde
Selection of antibodies
Immunochemically well-defined
Applicable on frozen sections
Consistent quality
Supply of adequate amounts
Preferably those used in ELISA
Tissue storage and fixation
Frozen tissue: store at $-80^{\circ}C$ or lower
Fixation of frozen sections: acetone, methanol or formaldehyde
Paraffin embedding after fixation with 4% buffered formaldehyde, standardised duration of fixation
Immunohistochemical staining procedure
Sensitive: avidin–biotin complex or detection technique of comparable sensitivity
Antigen retrieval procedures for paraffin sections
(Pre) treatment of sections with proteolytic enzymes
Microwave treatment using solutions of various composition and pH

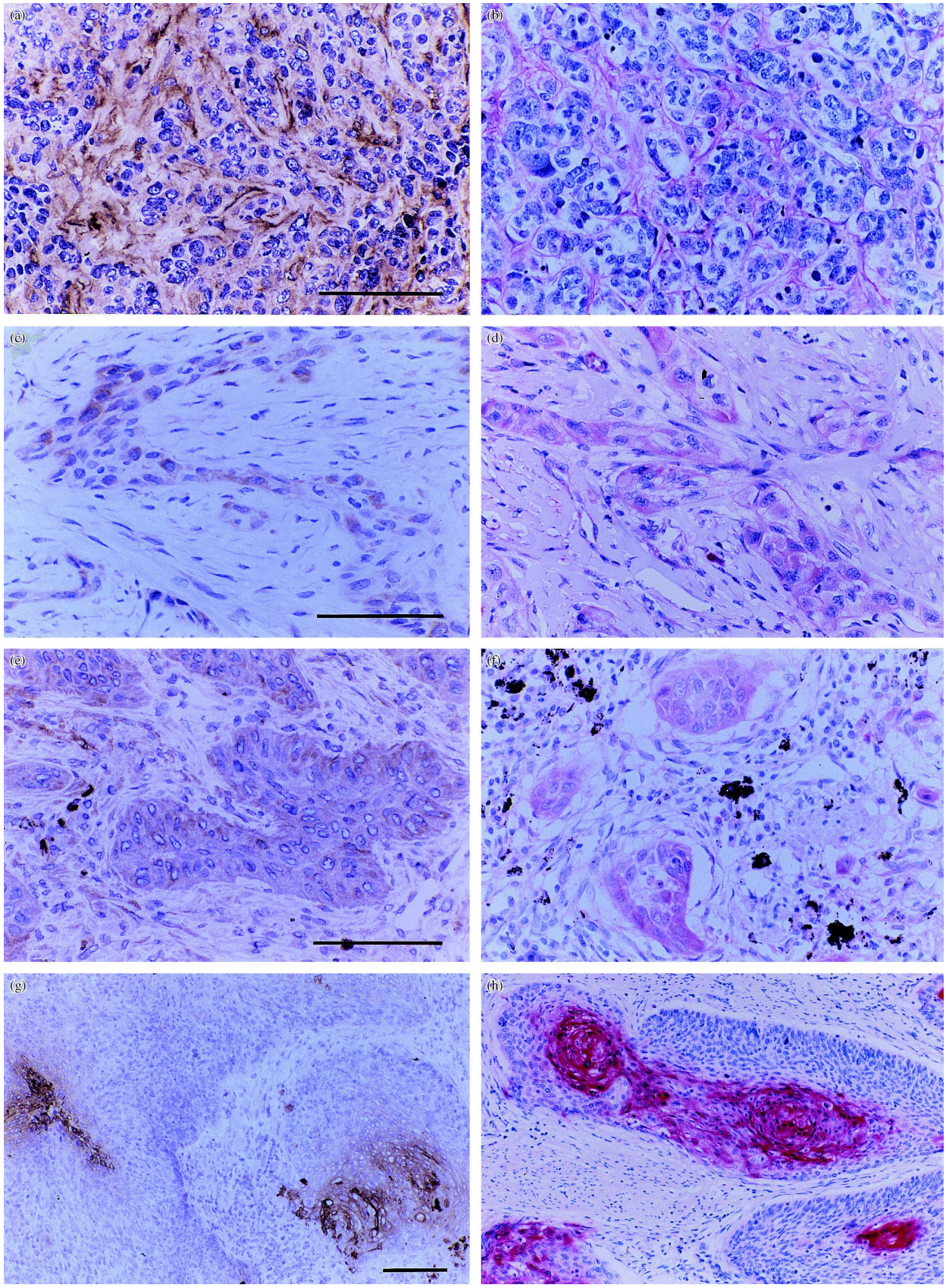

Figure 1. Staining for (a, b) urokinase-type plasminogen activator (uPA), (c, d) tissue-type plasminogen activator (tPA), (e, f) plasminogen activator inhibitor type 1 (PAI-1) and (g, h) plasminogen activator inhibitor type 2 (PAI-2) on frozen sections (a, c, e, g) and corresponding paraffin sections (b, d, f, h). (a and b) show positive stromal cells combined with negative tumour cells in a breast carcinoma stained for uPA. (c and d) show a lung carcinoma stained for tPA; there is mainly tumour cell positivity. (e and f) show a lung carcinoma stained for PAI-1 with tumour cell bound positivity. (g and h) present a cervical carcinoma stained for PAI-2. There is strong tumour cell positivity for PAI-2 in the central parts of the tumour fields, whereas the outer layers of tumour cell nests are negative. Bar = 100 μm; magnification of frozen and corresponding paraffin sections are the same.

under pressure cooking conditions in retrieval solutions of varying composition and pH may be helpful [25–30]. Also, cocktails of monoclonal antibodies recognising different epitopes on the same antigen may be used in order to obtain a stronger signal. With regard to the components of the PA system, several methods of pretreating paraffin sections to enhance epitope accessibility have been reported [20, 31–34]. Systematically optimised retrieval techniques for the different PA components have been studied recently [18]. Using such conditions, we have obtained distinct staining profiles with antibodies against uPA, tPA, PAI-1, PAI-2 and uPAR in human tumour lesions. Figure 1 shows the comparison of frozen and paraffin sections from the same lesion for different components.

QUALITY OF THE INTERPRETATION OF IMMUNOHISTOCHEMICAL STAINING RESULTS

Depending on the type of cancer, tumour cells, stromal cells and extracellular matrix may stain for the various components of the PA system. A scoring system should take this into account. Scoring systems used to date are based on the estimated proportion of lesional cells stained. Based on a large experience of evaluating the expression of several types of tumour-associated antigens we recommend the following distribution of proportion classes: 0% (absent), 1–5% (sporadic), 6–25% (local), 26–50% (occasional), 51–75% (majority), 76–100% (large majority). Using this approach for the expression of various antigens in tumour cells we have met a concordance of the score in about 90% of cases studied. In the remaining 10% of cases it should be possible to reach consensus on the score between the observers, during a joint histopathological examination. Regarding tumour cell staining, only those with an intensity + (dull), 2 + (clear) or 3 + (bright) are scored. Scoring is performed at intermediate power fields (×100) interchanged with selective inspection at high power (×250). Faint staining is neglected. As there may be small variations between different staining sessions it is better to perform these in relatively large batches including control tissues with a known staining pattern and staining intensity. Semiquantitative scoring of immunohistochemically stained tumour cells for the oestrogen receptor, in which both the percentage of stained nuclei and staining intensity were taken into account, was reported to be valuable for correlative inter-laboratory studies, although the scoring protocol used may not be required for diagnosis or prognosis [35]. For distinct nuclear staining, e.g. of steroid receptors and proliferation markers, image analysis may be a valid alternative to visual scoring [36]. However, in case of cytoplasmic and/or membranous staining this approach seems less practical.

Much less experience exists on the scoring of stromal staining. Since it is more difficult to estimate the percentage of positive stromal cells, a less accurate categorisation for stromal cells is proposed: stromal cells staining being absent, present in spare parts, or present abundantly. Moreover, the type(s) of stromal cells stained (e.g. fibroblasts, macrophages, microvascular cells) should be noted. In case of local staining of a lesion, it is recommended to mention the topographical area of staining, e.g. central part of the tumour, invasive front. Extracellular matrix staining, as seen with antibodies directed against PAI-1, is noted and described as sporadic, local or diffuse. Santavicca and colleagues [37] described a semiquantitative evaluation of stromelysin-3 expression in

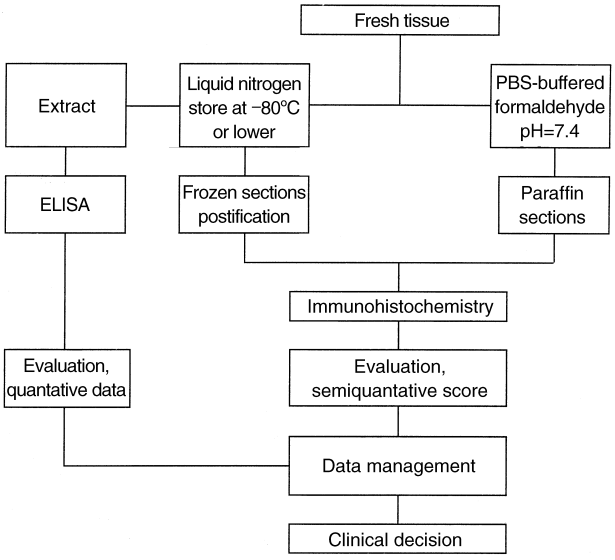

Figure 2. Flow chart illustrating an optimal use of tumour material.

paraffin-embedded tissue sections in breast carcinoma lesions. Their score comprised: (a) dispersion of fibroblastic cells stained; (b) proportion of fibroblastic cells stained; and (c) intensity of staining. It has to be determined which of these parameters might have prognostic value in breast cancer. Tumour vessels are another stromal element that harbour prognostic information in human tumours [38]. Most widely used is a protocol described by Weidner and associates [39], in which vessels are counted in the so called 'angiogenic hot spot'. However, the selection of the hot spot has been shown to be subject to observer variation [38]. In a recent study [40], we showed that image analysis offers an objective and more reproducible method to quantify tumour vascularity than manual counting of vessel profiles in the hot spot. Still, both methods were able to discriminate between the level of vascularisation in the two types of melanoma xenograft studied.

In order to use the scoring system on a wider scale, a learning set has to be prepared with striking examples of different staining patterns and different intensities. After a learning phase, a test set of other tissue sections should be stained, evaluated and scored. Only those histopathologists that reach an accordance of 90% with the score of the expert panel are qualified to score in the setting of assessment of prognosis. In approximately 10% of the lesions studied, the immunohistochemical staining results are validated by using other methods on the corresponding tissue sample. Ideally, the areas taken for these additional studies are microscopically selected. Confirmative techniques are ELISA, *in situ* zymography and on the RNA level reverse transcription–polymerase chain reaction and/or *in situ* hybridisation. These techniques can be performed on limited amounts of tissue. A future development useful for this purpose is the laser microdissection microscope [41].

CONCLUSION

In conclusion, we discussed an integral quality control system for the immunohistochemical evaluation of proteases and related components in tissue sections of tumour lesions obtained from patients with solid tumours. The stringent application of this quality control system should enable a

consistent assessment of the prognostic value of immunohistochemical detection of the PA components for cancer patients similar to that for ELISA [16, 17]. Optimal cooperation between clinical chemistry laboratories and departments of pathology should lead to a complementary use of ELISA and immunohistochemistry, respectively. A coordinated approach is illustrated in Figure 2. The use of ELISA is recommended in those cases in which extracts from fresh tumour lesions are available, immunohistochemistry when fixed tissue has to be used and localisation of a component is correlated with biological behaviour. The application of immunohistochemistry is recommended, but only in a setting where the conditions of the quality control system described can be met. One goal of the European BIOMED-1 Concerted Action is to determine whether immunohistochemical assessment of proteolytic factors is of clinical use in defining high risk cancer patients. This has already been shown by ELISA and suggested by immunohistochemistry in some instances [42–45]. In the context of clinical consequences, it is very important that an integrated quality control system is available.

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