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METHOD

Significance of Wet Autoclave Pretreatment in Immunohistochemistry

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Until recently the only way to rescue masked epitopes in routinely processed surgical pathological material was enzymatic digestion. The use of heat for antigen retrieval, first by microwave irradiation, represents an important breakthrough in immunohistochemistry. With the acceptance of microwave oven pretreatment, various modified techniques and alternative heating methods have also been proposed. Wet autoclave pretreatment for tissue proteolysis is a highly reliable alternative to the microwave antigen retrieval technique. It provides uniform heating of the slides, hence an even enhancement of staining intensity in a variety of formalin-sensitive antigens, and it also offers consistent interlaboratory results. The method has been introduced in routine diagnostic immunohistochemistry for the detection of estrogen- and progesterone receptors, L26-, Ki-67- and bcl-2 antigens and variable types of cytokeratins (1/5/10/11, 8, 13, 19). Experimentally, wet autoclaving can be used very successfully for the immunophenotyping of p53 and mdm2 expression, for the detection of adhesion molecules (CD44, integrins) and some anti-inflammatory molecules (annexins), among others. It has produced a substantial improvement in the visualisation of silver-stained nucleolar organizer regions- associated proteins (AgNORs) in routine paraffin sections and along with modified silver staining and standardized AgNOR parameters assessed by image analysis. Wet autoclaving-based AgNOR staining has been proposed by a European multicentric study group as the standardized method for AgNOR analysis in archival material. (Pathology Oncology Research Vol 2, No1–2, 71–77, 1996)

Key words: Wet autoclave pretreatment; Immunohistochemistry; AgNOR

Introduction

The term "antigen retrieval" has been introduced to describe different enzymatic and non-enzymatic techniques which can reverse the deleterious effect of formaldehyde fixation on tissue antigenicity.[1,8,13,14,31] The introduction of the *microwave oven*,[3,4,9] has radically obviated the old dogma, that excessive heat should not be used during tissue processing because of its denaturating effect. Through the use of microwave heating, many important markers became accessable for immunostaining in routine paraffin sections. It soon became apparent, however, that this method can produce inconsistent results as a function of different power levels, solution volumes and heating times. Besides, it is relatively labourous and its capacity (i.e. the number of slides that can be reliably heated in the oven at one time) is relatively small. The use of an *autoclave*,[35] as an alternative heat source can reduce most of the drawbacks of microwave pretreatment. A modified method: *wet autoclave pretreatment*[3] (WAP), has been proposed as a reliable alternative to both microwave oven heating and hydrated autoclaving in diagnostic immunohistochemistry. The advantages of WAP compared to microwave oven heating number at least three:

a) autoclaving ensures more even heating of the slides and allows the handling of a large number of sections with rather consistent results;
b) autoclaving can be applied without any significant damage to morphology and loss of sections;
c) the method is simple, reproducible and standardized.

The other important application of WAP is the visualisation of silver-stained nucleolar organizer regions-asso-

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ciated proteins (AgNORs) in routinely processed tissues.[21] The amount and distribution of AgNORs within interphase cell nuclei has been suggested as a means to estimate proliferation activity, as well as, the prognosis of different human malignancies.[17,11,20] WAP might exert its antigen demasking effect by extracting calcium and/or other divalent metal ions tightly complexed to formaldehyde-fixed proteins resulting in a second denaturation, or "renaturation" of proteins, including the antigens concerned.[18] Each methodical step with respect to section preparation, autoclave pretreatment, immunostaining, as well as silver staining of the AgNORs, quantification and controls have been described in detail.[3,21]

Some general considerations

a) WAP can be performed on all kinds of routinely formalin-fixed and paraffin-embedded tissues, irrespective of the duration of fixation (from 12 hours up to several years) or paraffin embedding (up to 10 years, or more).

b) To ensure a better adherence of tissue sections on glass slides, the use of poly-L-lysine- or silane coated-, or specially pretreated slides (e.g. SuperFrost Plus*, Menzel-Gläser, Germany – or equivalent) is required.

c) A 10-minute autoclave heating at 120°C, under 2-2,5 bar pressure, produces optimal staining quality with the majority of antibodies tested. In the case of suboptimal enhancement, a 5-minutes autoclaving followed by an intensified immunohistochemical detection (e.g. repeated incubation both with the secondary antibody and the tertiary enzyme-labelled complex and/or longer enzymatic development) is recommended. Autoclave pretreatment for AgNOR staining requires 20 minutes.

d) Both peroxidase, and alkaline-phosphatase-based detection systems can be used excellently following autoclave pretreatment. In our work a great variety of antibodies showed the unambigous advantage of WAP over microwave oven heating and/or non-pretreating protocols. We are using WAP routinely for the following antibodies: *against cell surface antigens* (CD44st, CD44v4, -v5, -v6, -v7-8, -v9, L26 [B lymphocyte marker]) *(Fig.1); against cytoplasmic antigens* (vimentin, bcl-2, *(Fig.2)* TGF-α, TGF-β, cytokeratins 1/5/10/11, -8, -13, -19); *against nuclear antigens* (Ki-67 *(Fig.3)*, p53 (CM1, PAb1801, PAb240, DO7), mdm2 *(Fig.4)*, waf-1, pRb, estrogen-, progesterone- and androgen receptors).

Methods

Preparation of sections and autoclave pretreatment: for immunohistochemistry

2 µm thick sections were cut and floated on poly-l-lysine-, or alternatively on silane-coated slides and heat fixed at 37°C overnight. Deparaffinization was carried out in xylene (2x10 minutes) followed by rehydration in a series of descending ethanol. From the last distilled water bath, slides were transferred into plastic Coplin jars filled with a citrate buffer solution (0.01 M citric acid monohydrate, pH 6.0). Coplin jars were covered with lids and wrapped in foil.

Autoclaving was carried out for 5-10 minutes at 120°C in a Gössner Laborautoklav GLA-40-2. After cooling down to room temperature over a period of 30 minutes, slides were briefly rinsed in a 0.05 M Tris-HCl buffer, pH, 7.4.

Immunostaining

Optimal dilutions of the primary antibodies were titrated on representative tissue sections following 10 minutes of autoclaving. Incubations with the primary antibodies were carried out overnight (14-16 hours) in a humidified chamber at 4°C. Subsequent to primary antibody incubations, a rabbit-anti-mouse or a mouse-anti rabbit bridging antibody (1:30, or 1:125 in RPMI, 30 minutes at room temperature; Dakopatts, Copenhagen, Denmark) and a monoclonal mouse-APAAP (alkaline phosphatase anti-alkaline phosphatase) complex (1:100 in RPMI; 60 minutes at room temperature, Dakopatts) were applied. For the primary antibody reactions, following incubation for 5-minutes in the autoclave (bcl-2 and Pab240), a "double" APAAP reaction was performed (10 minute repeats for both the bridging antibody and the APAAP complex). Enzyme development was carried out for 30 minutes at room temperature in a freshly prepared Fast Red solution containing naphthol-AS-MX-phosphate (alternatively New Fuchsin staining). For the detection of mdm2 immunoreactivity, an NBT/BCIP (nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate) chromogen solution was applied for 30-60 minutes in the dark at room temperature. Finally, sections were rinsed in tap water, counterstained with hematoxilin and mounted in Kayser's glycerine gelatine.

Preparation of sections and autoclaving for AgNOR staining

2 µm thick sections were floated on silane-coated slides. Dewaxing steps were identical to those for immunohistochemistry. Autoclaving was performed under the same conditions but for 20 minutes. Optimal staining results were obtained using a freshly prepared silver-staining solution containing one part 2% gelatine in 1% formic acid and two parts 25% aqueous silver nitrate solution at an incubation time of 25 minutes. Finally all sections were thoroughly rinsed in deionised water, dehydrated in graded ethanols, cleared in xylene and mounted with Entellan (all chemicals were purchased from Merck, Darmstadt, Germany).

Controls

Omission of primary antibodies was used as a negative control for each tissue block investigated in the test-phase of the study. Autoclave pretreatment was controlled by the

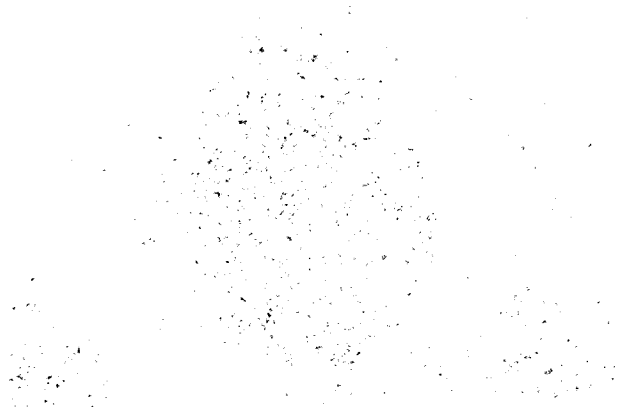

Figure 1. Immunohistochemical demonstration of B-lymphocytes using a monoclonal L26 antibody (Dako) in a routine paraffin section of a tonsil following wet autoclave pretreatment. (APAAP method, x65).

use of distilled water instead of sodium-citrate buffer as well as by omission of autoclave pretreatment both for immunohistochemistry and AgNOR staining. With some antibodies, MIB 1, Ki-67, anti-estrogen-receptor, anti-p53 (CM1-, PAb1801, PAb240, DO7) and anti-CD44 isoforms, a parallel microwave pretreatment was also performed on serial sections in a Brother Power Microwave oven at maximum output (~600W). Different incubation times were tested: either 2x5 minutes, as in the original microwave method (Cattoretti), for the p53 and CD44 stainings, 7x5 minutes for MIB 1 and Ki-67 reactions and 5x4 minutes for the estrogen receptor studies, according to the recommendations of the manufacturers of the respective antibodies. All microwave irradiations were performed in a 0.01 M sodium citrate buffer (pH 6.0). AgNOR staining was controlled by replacing citrate buffer with a 10 mM solution of EDTA (ethylene-diamine-tetra-acetic acid). Counting was performed by eye.

Figure 2. Immunoreactivity of a monoclonal anti-bcl-2 antibody (Dako) on a paraffin embedded tonsil section. (APAAP method, x65).

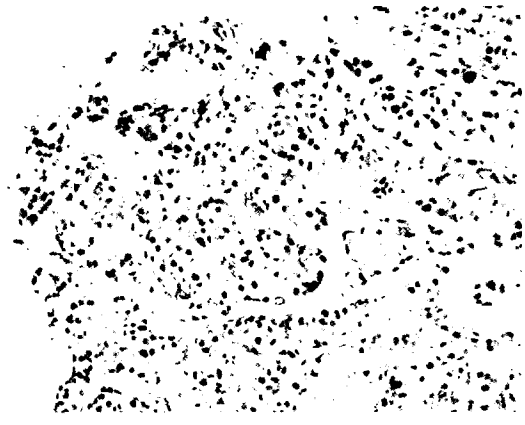

Figure 4. Immunoreactivity of a monoclonal anti-mdm 2 antibody on a paraffin embedded invasive ductal carcinoma of the breast. (NBT/BCIP development, x100).

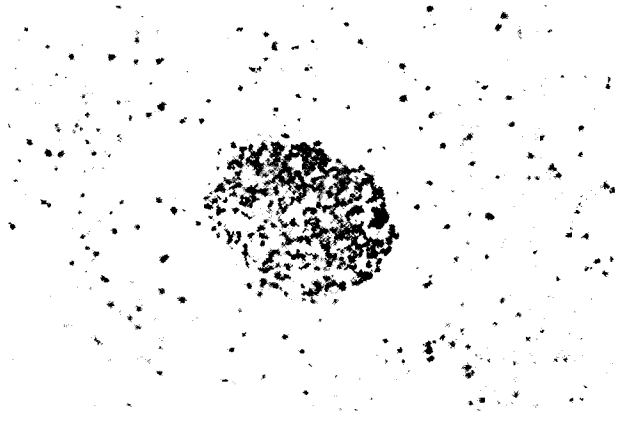

Figure 3. MIB 1 (monoclonal, Dianova) immunoreaction on a paraffin tonsil section (APAAP method, x65).

Some comments on our experience with WAP

Immunohistochemical reactions were semiquantitatively evaluated with respect to staining intensity and quantity of stained cells. The adequate histological and subcellular localization of the reactions has always been thoroughly analysed. No false negative or false positive, or non-specific immunoreactions have been observed following WAP, yet. Detailed description on the reactivity pattern of each antibody tested have already been published.[3,4,7,26] Essentially, autoclave pretreatment resulted in at least equal staining quality [e.g. ER detection in breast cancers with high estrogen receptor (ER) content, CM1, PAb1081, DO7, CD44 isoforms] or superior enhancement effect (e.g. MIB 1, Ki-67, PAb240, ER detection in breast cancers with low estrogen receptor content, vimentin) compared to microwave pretreatment. Morphology was

generally better preserved following autoclaving and floating off of sections was also less frequent. Autoclaving always produced superior staining quality compared to the non-pretreating protocol.

WAP enhanced the visualisation of AgNORs as a result of better discrimination of single intranucleolar "black dots".[21] Autoclave pretreatment significantly increases the detectable mean number of AgNORs per nucleus while area measurements were less influenced by autoclave pretreatment.

Comments on certain antibodies

WAP prior to immunostaining enables exposure of several antigens to immunohistochemistry which previously have been masked by routine formalin fixation and tissue processing. Some of those antigens are useful in routine diagnostic immunohistochemistry while others are targeted by research.

Determination of the *estrogen receptor* (ER) status is an important factor both for treatment decisions and prognosis of breast carcinoma patients. Great efforts have been expended over the last decade to overcome the need for fresh tumor tissue, which is essential for both biochemical ER demonstration and the widely used immunocytochemical assay (ERICA) applying the H222 monoclonal antibody (Abbott) on frozen sections. In a recent study, Bier *at al.*[7] analysed immunohistochemically the ER status of breast carcinomas on serial paraffin sections using WAP, microwave oven heating and enzymatic digestion with four different monoclonal antibody clones (H222, LH1, CC4-5, 1D5.26). They compared the results obtained from paraffin sections with ER status detected on frozen sections and with biochemical analysis. It was pointed out that the sensitivity and specificity of immunohistochemical ER detection was highest when the 1D5.26 antibody was used following WAP in paraffin sections, especially when low ER content was detected biochemically *(Figs.5a,b)*.

Figure 6. *Immunohistochemical demonstration of p53 expression in normal and neoplastic oral mucosa in paraffin material following wet autoclave pretreatment. (monoclonal Pab240, Medac, Germany, APAAP reaction, x65).*

One of the most extensively studied molecules in biomedicinal research is the tumor suppressor gene product, p53.[15] Immunohistochemistry for p53 protein expression has been proposed as a valid screening method for predicting underlying mutations in the p53 gene in a variety of human malignancies, whereas in other tumor types this correlation could not be confirmed.[2,6,10,12] A growing body of evidence on the significance of extragenetical events inactivating the function of the wild-type p53 protein without mutations of the gene renders p53 immunohistochemical studies on the protein level to be an independent field of research. However, p53 immunoreactivity in routine paraffin sections largely depends on the type of antibody and pretreatment method used. Recently, we applied a panel of four anti-p53 antibodies (CM-1, PAb1801, DO7, PAb240) on a series of routine paraffin sections of oral squamous cell carcinomas in order to compare the effectiveness of WAP and microwave oven irradiation for antigen retrieval in contrast to non-pretreated immunos-

Figure 5 a. b. *Immunohistochemical oestrogen receptor demonstration in serial paraffin sections of a breast carcinoma following 5* ***(a)*** *microwave- and 5* ***(b)*** *wet autoclave pretreatment (monoclonal 1D5.26 antibody, Dianova, Germany, APAAP reaction, x65).*

Figure 7. Immunohistochemical detection of the CD44 v6 isoform in paraffin embedded oral squamous cell carcinoma. (monoclonal VFI18 antibody, Serca, Germany, APAAP reaction, x100).

taining.[26] The results showed that WAP was significantly superior with all anti-p53 antibodies used when compared to conventional immunohistochemistry without pretreatment. Furthermore, it yielded better results than microwave irradiation for antigen demasking using the PAb240 antibody. The reliability and reproducibility of p53 antigen retrieval by WAP in routinely processed archival material was also proved in oral squamous cell carcinomas and adjacent non-tumorous mucosa[27] *(Fig.6)*.

Other regulating proteins could also be good targets for WAP. A possible relationship between p53 and mdm2[17,39] oncoprotein expression has been reported recently in colorectal carcinomas,[24] in thyroid carcinomas,[33] and has been observed in oral squamous cell carcinomas, as well.[27] In all three tumor types, an immunohistochemical colocalisation of mdm2 and p53 proteins was detected in a portion of malignancies providing further evidence that the mdm2 gene product interacts with p53 protein. This can result in an immunohistochemically detectable accumulation of p53 without underlying mutations of the p53 gene in some cases.

Programmed cell death (apoptosis) is an other "hot spot", with the bcl-2 gene family playing a dominant role in the regulation of the process. The bcl-2 protein was found to inhibit apoptosis. However, its biochemical function is not fully understood yet.[38] Recently, Öfner *et al.*[22] reported an archival immunohistochemical study in a retrospective setting of human colorectal carcinomas, where the use of wet autoclave antigen retrieval enabled the immunohistochemical detection of bcl-2 protein in routinely processed paraffin material. They found that bcl-2 protein overexpression is associated with favourable clinical outcome of patients and represents an independent marker of prognosis in colorectal cancer. The authors proposed bcl-2 immunophenotyping as an additional tool to contribute to the future clinical management of such patients.

Preliminary data indicate that, following WAP, several *isoforms of the* CD44 adhesion molecule-family and some anti-inflammatory molecules (annexins) can be targeted by immunohistochemistry in routinely processed tissues, as well. *(Fig.7)*

The *argyrophylic nucleolar organiser regions-associated proteins* (AgNORs) correspond to a set of intranucleolar acidic non-histone proteins – two of them have been identified recently (nucleolin and B23)[12,16] – which regulate the transcription of preribosomal mRNA. AgNORs can be selectively stained by a silver colloid technique[30] and visualized as dark intranucleolar dots under the light microscope. Until recently, a large number of retrospective AgNOR studies were performed on routinely formalin-fixed and paraffin-embedded tissues, both for diagnosing and even for prognostication of different human malignancies. All of these approaches lacked interobserver- and interlaboratory reproducibility. Also the quality of staining, which is essentially influenced by fixation and silver

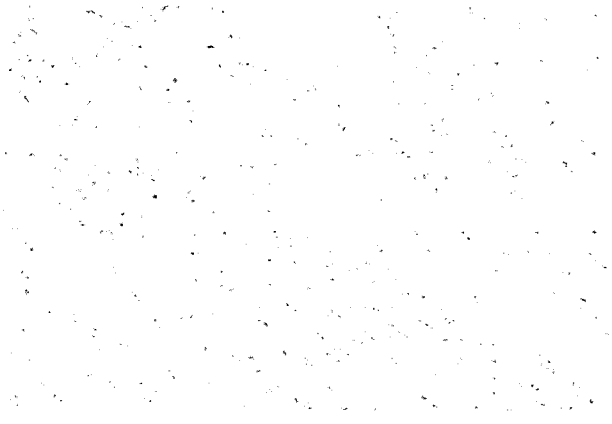

Figure 8. Standardized AgNOR silver-staining following wet autoclave pretreatment in a paraffin section of an oral squamous cell carcinoma. (x100).

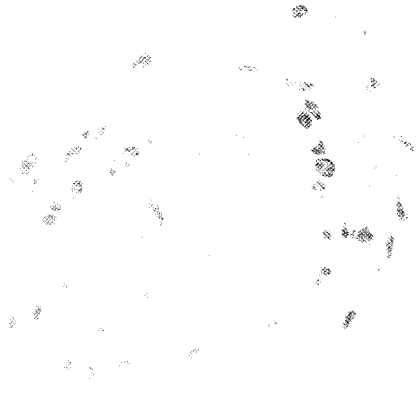

Figure 9. Simultaneous demonstration of AgNORs and MIB1 immunoreactivity in a paraffin embedded oral squamous cell carcinoma section, following wet autoclave pretreatment.

staining protocol, was rather poor.[3] More recently, a novel technique for AgNOR staining and evaluation has been introduced in archival material based on wet autoclave pretreatment[21] with excellent staining quality and high reproducibility. Therefore, an international study group has proposed this to be the standardized method for archival AgNOR studies. By using this standardized AgNOR protocol, the quantity of AgNORs was found to be an independent factor of prognosis in breast- and colorectal cancer.[23,24] Preliminary data show similar correlations in oral cancers.* As a measure of metabolic activity of a cell and of the velocity of the cell cycle, AgNORs were found to be expressed at the highest amount at the invasive front of oral squamous cell carcinomas signaling a subgroup of tumor cells with probable elevated malignancy potential.[29] *(Fig.8)* Using WAP, simultaneous demonstration of AgNORs and MIB 1 immunoreactivity can be performed within the same histological section, allowing analysis of AgNOR quantity in cycling cells separate from resting ones. This has outstanding potential for studying cell proliferation in paraffin material *(Fig.9)*.

As a corollary, WAP of formalin-fixed and paraffin-embedded sections proved to be a sensitive and reliable method of antigen demasking and AgNOR-protein retrieval. The technique is simple, reproducible and standardized. The main disadvantage of the method is the expensive and cumbersome nature of the autoclave. Various alternative heating methods have also been proposed and tested from a domestic pressure cooker,[19] to the application of a pressure cooker within a microwave oven.[36] Taken together, results suggest that the effectiveness of antigen recovery is a function of time and temperature and depends both on the nature and the pH of the antigen retrieval solution, which can be variable for different antigens. The method providing the greatest reliability in protein retrieval in standardized conditions will surely be welcomed. Until now, no alternative heating methods could surpass WAP in this respect.

References

1. *Abbondanzo SL, Allred DC, Lampkin S and Banks PM:* Enhancement of immunoreactivity among lymphoid malignant neoplasms in paraffin-embedded tissues by refixation in zinc sulfate-formalin. Arch Pathol Lab Med 115:31-33, 1991.
2. *Ahomadegbe JC, Barrois M, Fogel S, Le Bihan ML, Douc-Rasy S, Duvillard P, Armand JP and Rion G:* High incidence of p53 alterations (mutation, deletion, overexpression) in head and neck primary tumors and metastases; absence of correlation with clinical outcome. Frequent protein overexpression in normal epithelium and in early non-invasive lesions. Oncogene 10:1217-1227, 1995.
3. *Bánkfalvi A, Navabi H, Bier B, Böcker W, Jasani B and Schmid KW:* Wet autoclave pretreatment for antigen retrieval in diagnostic immunohistochemistry. J Pathol 174:223-228, 1994.
4. *Bánkfalvi A, Riehemann K, Öfner D, Checci R, Morgan JM, Piffkó J, Böcker W, Jasani B and Schmid KW:* Feuchtes Autoklavieren: der einfachere Weg zur Antigendemaskierung. Pathologe 15:345-349, 1994.
5. *Bánkfalvi A, Öfner D, Piffkó J and Schmid KW:* Nucleolar organizer regions in lining epithelium adjacent to squamous cell carcinoma of human oral mucosa.(Correspondence) Cancer 74:3245-3246, 1994.
6. *Bartek J, Bartkova J, Vojtesek B, Staskova Z, Lukas J, Rejthar A et al:* Aberrant expression of the p53 oncoprotein is a common feature of a wide spectrum of human malignancies. Oncogene 6:1699-1703, 1991.
7. *Bier B, Bánkfalvi A, Grote L, Blasius S, Öfner D, Böcker W, Jasani B, Schmid KW:* Wet autoclave pretreatment for immunohistochemical demonstration of oestrogen receptors in routinely processed breast carcinoma tissue. Histochemical J 27:148-154, 1995.
8. *Brandtzaeg P:* Tissue preparation methods for immunohistochemistry. In: Bullock GR, Perusz P eds. Techniques in Immunocytochemistry. Vol 1. Academic Press, 1-75, 1982.
9. *Cattoretti G, Pileri S, Parravicini S, Becker MHG, Poggi S, Bifulco C, Key G, D'Amato L, Sabattini E, Fendale E, Reynolds F, Gerdes J and Rilke F:* Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. J Pathol 171:83-98, 1993.
10. *Cesarman E, Inghirami G, Chadburn A and Knowles DM:* High levels of p53 protein expression do not correlate with p53 gene mutations in anaplastic large cell lymphoma. Am J Pathol 143:1389-1397, 1993.
11. *Derenzini M, Nardi F and Farabegoli F:* Distribution of silver stained interphase nucleolar organizer regions as a parameter to distinguish neoplastic from non-neoplastic reactive cells in human effusions. Acta Cytol 33:491-498, 1989.
12. *Dockhorn-Dworniczak B, Schröder S, Dantcheva R et al.:* The role of the p53 tumor suppressor gene in human thyroid cancer. Exp Clin Endocrinol 101:39-46, 1993.
13. *Finley JCW and Petrusz P:* The proteolytic enzymes for improved localization of tissue antigens with immunohistochemistry. In Bullock GR, Perusz P eds. Techniques in Immunocytochemistry. Vol 1. Academic Press, 239-249, 1982.
14. *Kitamoto T, Ogomori K, Tateishi J and Prusiner SB:* Formic acid pretreatment enhances immunostaining of cerebral and systemic amyloidosis. Lab Invest 57:331-33, 1987.
15. *Levine AJ, Momand J and Finlay CA:* The p53 tumor suppressor gene. Nature 351:453-456, 1991.
16. *Lischwe MA, Smetana K, Olson MOJ and Busch H:* Proteins C23 and B23 are the major nucleolar silver staining proteins. Life Sci 25:701-708, 1979.
17. *Mohmand J, Zambetti GP, Olson DC, George D and Levine AJ:* The mdm2 oncogene product forms a complex with the p53 protein and inhibits p53 mediated transactivation. Cell 69:1237-1245, 1992.
18. *Morgan JM, Navabi H, Schmid KW and Jasani B:* Possible role of tissue-bound calcium ions in citrate-mediated high-temperature antigen retrieval. J Pathol 174:301-307, 1994.
19. *Norton AJ, Jordan S and Yeomans P:* Brief, high-temperature heat denaturation (pressure cooking): a simple and effective method of antigen retrieval for routinely processed tissues. J Pathol 173:371-379, 1994.

* *(Piffkó et al.* unpublished)

20. *Öfner D, Tötsch M, Sandbichler P, Hallbrucker C, Margreiter R, Mikuz G and Schmid KW:* Silver stained nucleolar organizer region proteins (Ag-NORs) as a predictor of prognosis in colonic cancer. J Pathol 162:43-49,1990.
21. *Öfner D, Bankfalvi A, Riehemann K, Bier B, Böcker W and KW Schmid:* Wet autoclave pretreatment improves the visualisation of silver stained nucleolar organizer region associated proteins (AgNORs) in routinely formalin fixed and paraffin-embedded tissues. Mod Pathol 7:946-950, 1994.
22. *Öfner D, Riehemann K, Maier H, Riedmann B, Nehoda H, Tötsch M, Böcker W, Jasani B and Schmid KW:* Immunohistochemically detectable bcl-2 expression in colorectal carcinoma: correlation with tumour stage and patient survival. Br J Cancer 72:981-985, 1995.
23. *Öfner D, Riedmann B, Maier H, Hittmair A, Rumer A, Tötsch M et al.:* Standardized staining and analysis of argyrophilic nucleolar organizer region associated proteins (AgNORs) in radically resected colorectal adenocarcinoma – correlation with tumour stage and long-term survival. J Pathol 1995:175:441-8.
24. *Öfner D, Maier H, Riedmann B, Holzberger P, Nogler M, Tötsch M, Bánkfalvi A, Winde G, Böcker W and Schmid KW:* Immunohistochemically detectable p53 and mdm2 oncoprotein expression in colorectal carcinoma: prognostic significance: J Clin Pathol Mol Pathol 48: M12-M16, 1995.
25. *Öfner D, Bier B, Heinrichs S, Berghorn M, Dünser M, Hagemann HA et al.:* Demonstration of silver-stained nucleolar organizer regions associated proteins (AgNORs) after wet autoclave pretreatment in breast carcinoma. Breast Cancer Research and Treatment (in press).
26. *Piffkó J, Bánkfalvi A, Öfner D, Joos U, Böcker W and Schmid KW:* Immunohistochemical detection of p53 protein in archival tissues from squamous cell carcinomas of the oral cavity using wet autoclave antigen retrieval. J Pathol 176:69-75, 1995.
27. *Piffkó J, Bánkfalvi A, Öfner D, Berens A, Tkotz T, Joos U, Böcker W and Schmid KW:* Expression of p53 protein in oral squamous cell carcinomas and adjacent non-tumorous mucosa of the floor of the mouth: an archival immunohistochemical study using wet autoclave pretreatment for antigen retrieval. J Oral Path Med 24:337-342, 1995.
28. *Piffkó, J, Bánkfalvi A, Öfner D, Tötsch M, Berens A, Joos U, Böcker W and Schmid KW:* Immunohistochemically detectable p53 and mdm2 expression with relation to proliferation in head and neck cancer. (submitted).
29. *Piffkó, J, Bánkfalvi A, Öfner D, Rasch D, Joos U, Böcker W and Schmid KW:* Standardized demonstration of silver stained nucleolar organizer regions (AgNORs) associated proteins in archival oral squamous cell carcinomas and adjacent non-neoplastic mucosa. (submitted).
30. *Ploton D, Menager M, Jeannesson P, Himber G, Pigeon F and Adnet JJ:* Improvement in the staining and in the visualization of the argyrophilic proteins of the nucleolar organizer region at the optical level. Histochem J 18:5-14, 1986.
31. *Rindi G, Buffa R, Sessa F, Tortora O and Solcia E:* Chromogranin A, B and C immunoreactivities of mammalian endocrine cells. Histochemistry 85:19-28, 1986.
32. *Roussel P, Belenguer P, Amalric F and Hernendez-Verdun D:* Nucleolin is an Ag-NOR protein; this property is determined by its amino-terminal domain independently of its phosphorylation state. Exp Cell Res 203:259-261,1992.
33. *Schmid KW, Bankfalvi A, Mücke S, Öfner D, Riehemann K, Schröder S, Stücker A, Tötsch M and Dockhorn-Dworniczak B:* Possible relationship of p53 and Mdm2 oncoprotein expression in thyroid carcinoma. Endocrin Pathol (in press).
34. *Shi SR, Key ME and Kalra KL:* Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem 39:741-74, 1991.
35. *Shin RW and Iwaki T, Kitamoto T, Tateishi J:* Hydrated autoclave pretreatment enhances tau immunoreactivity on formalin-fixed normal and Alzheimer's disease brain tissues. Lab Invest 64:693-702, 1991.
36. *Taylor CE:* A comparative study of antigen retrieval heating methods. CAP Today, September, 1995.
37. *Trere D, Pession A and Derenzini M:* The silver-stained proteins of interphasic nucleolar organiser regions as a parameter of cell duplication rate. Exp Cell Res 184:131-137, 1989.
38. *Tsujimoto Y, Cossman J, Jaffe E and Croce C:* Involvement of the bcl-2 gene in human follicular lymphoma. Science 228:1440-1443, 1985.
39. *Wu X, Bayle JH, Olson D and Levine AJ:* The p53-mdm2 autoregulatory feedback loop. Genes Dev 7:1126-1132, 1993.