Histopathology 2010, **57**, 725–733. DOI: 10.1111/j.1365-2559.2010.03684.x

Immunohistochemical detection of arginine methylated proteins (MeRP) in archival tissues

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Date of submission 3 December 2009
Accepted for publication 5 March 2010

Vezzalini M, Aletta J M, Beghelli S, Moratti E, Della Peruta M, Mafficini A, Mojica W D, Mombello A, Scarpa A & Sorio C

(2010) *Histopathology* **57**, 725–733

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Aims: To (i) determine whether methylarginine-specific antibodies can be employed for standard immunohistochemical analysis of paraffin-embedded tissues, (ii) analyse methylarginine expression in normal and neoplastic tissues and (iii) correlate methylarginine expression with that of protein arginine methyltransferase (PRMT1), the predominant cellular arginine methyltransferase.

Methods and results: Immunohistochemistry of normal and cancer tissues was performed utilizing three commercial polyclonal antibodies: anti-methylarginine-specific antibody (anti-mRG) raised against a methylarginine peptide, Control antibody (anti-RG), a control antiserum raised against a corresponding arginine peptide without any methylated residues and anti-PRMT1. Nuclear and/or cytoplasmic methylarginine expression was detected in all keratinized and non-keratinized epithelia. A preliminary survey of a series of thyroid, pancreatic, colonic and gastric cancers identified a different pattern of methylarginine expression in comparison with normal tissue. A correlation between methylarginine staining and PRMT1 expression was found in all normal and cancer tissues analysed.

Conclusion: Methylarginine-specific antibodies are capable of recognizing methylarginine proteins (MeRP) in paraffin-embedded tissues. Methylarginine proteins are expressed widely and show differences in subcellular localization in various organs and neoplastic conditions. The efficient detection of methylproteins by standard immunohistochemistry provides a new tool to investigate the role of methylarginine proteins (MeRP) in biological processes including carcinogenesis.

Keywords: cancer, methylarginine, normal tissue, PRMT, immunohistochemistry

Abbreviations: AM, alveolar macrophage; GAR, glycine–arginine-rich; MeRP, methylarginine proteins; mRG, methylarginine-reactive protein; PRMT, protein arginine methyltransferase

Introduction

Proteins containing methylarginine residues have been implicated in a wide variety of human diseases, but the detection of methylarginine proteins (MeRP) in normal and pathological tissue specimens has not been reported.

The post-translational methylation of arginine has been described for proteins that exert an important role in transcriptional regulation.[1–3] The modification of arginine side chain guanidino groups is quantitatively one of the most extensive post-translational modifications of proteins in mammalian cells.[4,5] The protein arginine methyltransferase (PRMT) family of enzymes

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is responsible for the methyltransfer reaction using S-adenosyl-L-methionine as the methyl donor. These enzymes catalyse the formation of monomethylarginine and asymmetrical or symmetrical dimethylarginine in mammalian cells. Six genes with PRMT catalytic subunits are known to date (*PRMT1*, *3*, *4*, *5*, *6* and *8*) and three other genes encode sequence-related proteins with possible methyltransferase activities (*PRMT2*, *7* and *9*).[6,7] The number of distinct modified proteins is also diverse.[8] Further diversity of the methylation reactions occurring in living cells is added by alternative splicing mechanisms.[9] Individual members of the PRMT family differ in abundance, activity, substrate specificity and intracellular localization, indicating non-redundant roles *in vivo*. The predominant member of the PRMT family, PRMT1, is an essential enzyme responsible for approximately 85% of all arginine methylation reactions in human and mouse cells.[10–12] PRMT1 generally recognizes arginines within a glycine–arginine-rich (GAR) region, a motif present in many RNA- and DNA-binding proteins.

Based on the rapid expansion of biomedical research implicating methylarginine proteins in gene expression and other fundamental biological functions, the study of protein arginine methylation holds great promise for extending our understanding of human developmental and pathological processes.[13,14] Antibodies have been developed that recognize specifically a canonical methylarginine peptide sequence and the major arginine methyltransferase in eukaryotic cells, PRMT1;[15,16] however, a method for rapid screening of the process in biological tissues is lacking. Although Western blotting is a sensitive and quantitative assay that has been used to assess methylation status, it is not suited for high-throughput analysis.

Materials and methods

TISSUES

Formalin-fixed paraffin-embedded human normal and cancer tissues were retrieved from the archives of the Department of Pathology of the University of Verona.

ANTIBODIES

The immunoglobulin (IgG)-purified rabbit antibodies used in this work have been described previously.[15,16] They were polyclonal rabbit anti-mRG, anti-RG (CH3 BioSystems LLC, Buffalo, NY, USA) and anti-PRMT1 IgG purified by Protein A Sepharose chromatography[17] (a generous gift from Dr Harvey Herschman, UCLA).

WESTERN BLOTTING

Human normal tissues were frozen in liquid nitrogen vapours and stored at −80°C. Fifteen to thirty 20-μm cryostat sections were lysed in buffer containing 50 mM Tris pH 7.4, 1% Triton X-100, 150 mM sodium chloride (NaCl), 200 μM sodium orthovanadate (Na_3VO_4), 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 1 mM sodium fluoride (NaF), 1× final concentration complete EDTA-free protease inhibitor cocktail tablets (Roche, Mannheim, Germany) and processed for Western blotting, as described previously.[16]

IMMUNOHISTOCHEMISTRY

Four-μm-thick formalin fixed-paraffin-embedded sections were placed on polylysinated slides. Antigen retrieval was performed in 10 mM citrate buffer, pH 6.0, heated in a microwave at 360 W for 20 min. All subsequent incubations were performed at room temperature: endogenous peroxidase and non-specific sites were blocked by incubation with 3% H_2O_2 and 10% goat serum in phosphate-buffered saline (PBS)/0.05% Tween-20 for 15 min. Sections were then incubated with anti-mRG (8 μg/ml), anti-PRMT1 (12 μg/ml) or anti-RG (8 μg/ml), for 2 h, washed three times with PBST (PBS/0.2% Tween-20) and incubated for 40 min with the appropriate secondary antibodies: EnVision+ System – horseradish peroxidase (HRP)-labelled polymer antirabbit (DakoEnVision+®, peroxidase, Dako, Glostrup, Denmark). After three washes with PBST, diaminobenzidine (DAB; DakoCytomation liquid DAB+ substrate chromogen system) was used as peroxidase substrate for 10 min.

Results

Western blot analysis and immunohistochemistry related to methylarginine proteins were performed utilizing anti-mRG, anti-RG and anti-PRMT1 antisera.

mRG EXPRESSION IN NORMAL HUMAN TISSUES ANALYSED BY WESTERN BLOTTING

Western blot analysis of seven normal tissues showed a wide diversity of immunoreactive methylarginine proteins of molecular masses, ranging from 30 to 250 kD detected by anti-mRG (Figure 1, upper panel). Although the pattern of reactive protein bands was generally similar, there were perceptible qualitative and quantitative differences among the different tissues with regard to specific protein bands. The control

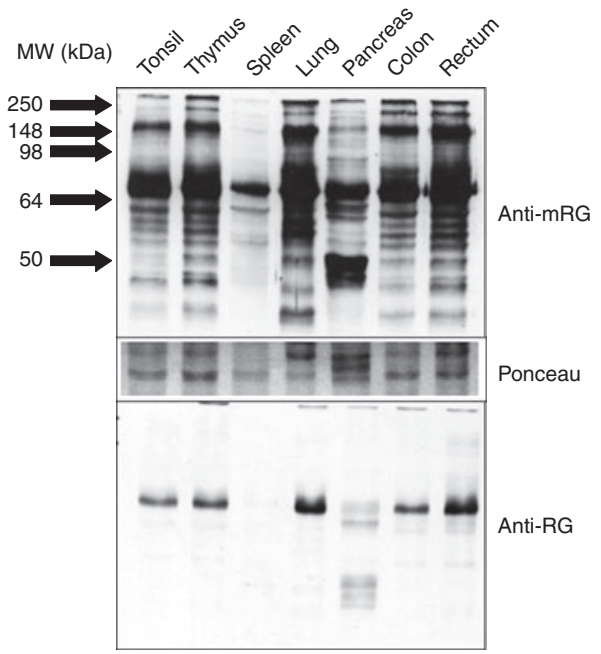

Figure 1. Methylarginine expression in normal tissues by Western blotting. Normal human tissues stained with anti-methylarginine-specific antibody (anti-mRG) (upper panel) and control anti-RG (lower panel) antibodies. Ponceau stain corresponding to the region of the blot between 50 and 70 kDa (middle panel) demonstrates comparable protein loads. Molecular weight markers are shown on left. Multiple bands are present in most tissues; note the relatively lower signal present in spleen and the presence of a major band in the 50 kDa range in pancreas. Control (anti-RG) antibody binds to a predominant band in the 70 kDa range weaker than that seen with the anti-mRG antibody.

antibody, anti-RG, raised against the non-methylated form of the peptide used to generate anti-mRG, gave dramatically different results with recognition of a predominant 70 kDa protein in most tissues (Figure 1, lower panel).These results confirmed the previously demonstrated specificity of anti-mRG.[16]

mRG AND PRMT1 IMMUNOHISTOCHEMICAL EXPRESSION IN NORMAL TISSUES

Specific nuclear and cytoplasmic mRG immunoreactivity was detected in epithelial and endocrine cells of various organs (Table 1).

mRG AND PRMT1 IN EPITHELIA

mRG was expressed in all the epithelia of the gastrointestinal tract, and most cells of the genitourinary system. mRG expression was also found in breast, ducts of parotid and sweat glands, acini and ducts of pancreas, bronchioli and pneumocytes. Inconsistent immunoreactivity was detected in fibroblasts. PRMT1 expression was generally coincident with that of mRG in the different epithelia. The exception was the PRMT1 signal in liver, which was only cytoplasmic with no nuclear staining, and fainter than that of mRG (Figure 2A,B).

mRG AND PRMT1 IN HAEMATOPOIETIC AND LYMPHOID TISSUES

Bone marrow myeloid precursors expressed mRG at variable levels, while erythroid precursors as well as megakaryocytes were negative (not shown). There was consistently strong immunoreactivity associated with lymphoid germinal centres (Figure 2B). In thymus, thymocytes stained only with anti-mRG antibody and PRMT1 was undetectable, while macrophages stained strongly with both antibodies (Figure 3). Macrophagic elements in spleen and lymphoid follicles of the tonsil and appendix expressed high levels of both mRG and PRMT1. By contrast, lung alveolar macrophages expressed high levels of mRG but lacked PRMT1 expression (Figure 3).

mRG AND PRMT1 IN ENDOCRINE TISSUES

There was high mRG expression in the whole adrenal gland (cortex shown in Figure 2B), thyroid (Figure 4) and islets of Langerhans (Figure 5), as well as other endocrine tissues. The signal intensity of methylarginine in each of these tissues was highest in nuclei.

mRG AND PRMT1 IN SELECTED TUMOURS

Looking for possible altered mRG expression associated with neoplasia, samples of epithelial (colonic, gastric and breast) and endocrine (thyroid and pancreatic) tumours were investigated. The comparison among various cancer histotypes summarized in Table 2 revealed that most breast cancers showed a high level of mRG with only a minority of cases (4.6%) showing a low level of staining; thyroid papillary cancers were always immunopositive; the overall reactivity of colonic, gastric and pancreatic neoplasms was approximately 80% positive. Various patterns of immunoreactivity were detected: cytoplasmic, Golgi-like, both nuclear and cytoplasmic and low/undetectable staining (Figure 6). These patterns appeared different to those observed in the corresponding normal tissues. We questioned whether PRMT1 expression could explain these patterns, as we observed a correlation between mRG staining and PRMT1 expression in normal tissues. We performed this analysis in pancreatic endocrine tumours (PET), where we found that the

Table 1. Immunohistochemical expression of methylarginine-reactive protein (mRG) and protein arginine methyltransferase (PRMT1) in various tissues and cell types

Tissue	Cell type	mRG	PRMT1
Skin	Squamous epithelium	+	+
	Sweat glands	ND	ND
Parotid	Duct epithelia	+	+
	Acini	–	–
Stomach	Foveolar epithelium	+	+
	Gastric glands	+	+
Duodenum	Surface epithelium	+	+
	Brunner glands	+	+
Small bowel	Surface epithelium	+	+
Appendix	Epithelium	–	–
Large bowel	Surface epithelium	+	+
Liver	Ductules	+	±
	Hepatocytes	+	±
Gall bladder	Epithelium	+	+
	Acini	+	+
Pancreas	Ducts	+	+
	Ductules	+	+
	Islets of Langerhans	+	+
Thyroid	Thyrocytes	+	+
	Parafollicular cells	+	+
	Colloid	–	–
Parathyroid	Cells	+	+
Adrenal	Cortex	++	+*
	Medulla	±	±
Bronchus	Epithelium	+	+
	Mucinous glands	–	ND
	Serous glands	+	ND
Lung	Pneumocytes type I	+	+
	Pneumocytes type II	+	+
	Alvolar macrophages	+	–
Kidney	Tubules	+	+
	Glomeruli	–	–
Ureter	Epithelium	+	+**
	Smooth muscle	±	±
Testis	Germ cells	+	+
	Leydig	++	–
Prostate	Epithelial cells	+	ND
	Stromal cells	±	ND
Spermatic ducts	Epithelium	+	+
	Smooth muscle	–	–
Ovary	Stroma	+	ND
Fallopian tube	Serous epithelium	+	ND
Uterus	Epithelium	+	+
	Stroma	±	±
Placenta	Cytotrophoblast	+	+
	Syncytiotrophoblast	–	–
	Endothelium	+	+
Breast	Duct epithelia	+	+
	Lobular epithelia	+	+
	Endometrium	+	+
Bone marrow	Megakaryocytes	–	ND
	Myeloid cells	+	ND
	Erythroid precursors	–	ND
	Macrophages/DC	+	ND
Thymus	Lymphoid cells	+	ND
	Epithelial cells	+	ND
Lymphoid follicle	Germinal centre	+	+
	Mantle	–	–
	Mantle zone	+	+
	FDC	+	+

+, Positive; ++, strongly positive; ±, faint positivity; ND, not done; *only nuclei; **only cytoplasm; FDC, follicular dendritic cells.

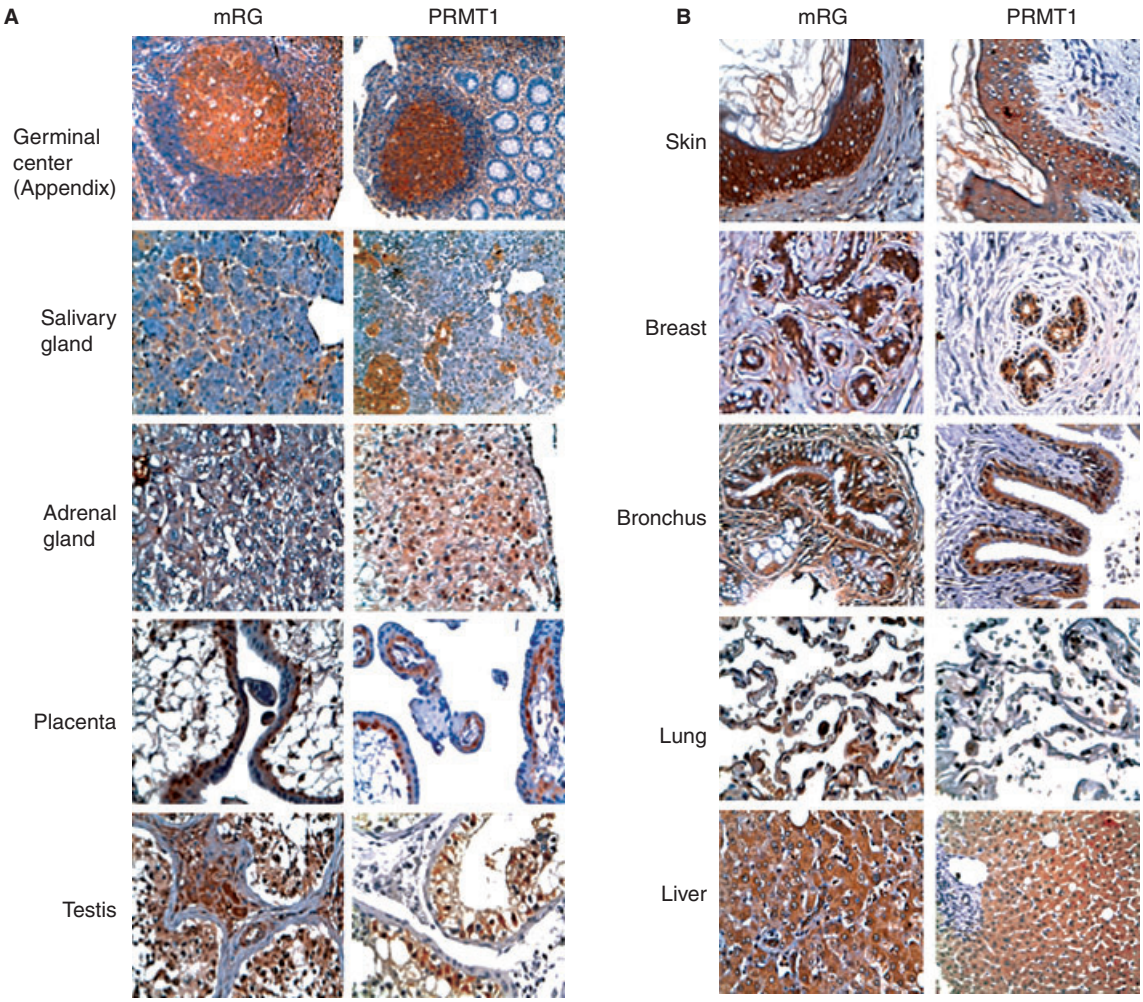

Figure 2. **A**, **B**, Methylarginine and protein arginine methyltransferase (PRMT1) expression in normal tissues. The tissues stained are indicated on the left. Note the correspondence between the cells that stain positive for methylarginine-reactive protein (mRG) and PRMT1.

immunoreactivity for mRG corresponded to the immunoreactivity for anti-PRMT1 in nine of 10 cases (seven positive and two negative cases for both antigens). This result suggested that PRMT1 expression was responsible for the majority of the mRG signal detected. The close association between the mRG and PRMT1 staining patterns implies that reduced protein arginine methylation represents a probable molecular explanation for the differential staining in the patient specimens.

Discussion

We have reported the application of immunohistochemistry to detect arginine methylated proteins (MeRP) in archival paraffin-embedded tissues, which provides rapid, semi-quantitative, subcellular cytological and histological data on the methylarginine status in physiological and pathological conditions.

Protein arginine methylation is involved in fundamental cellular functions including gene regulation, DNA repair, intracellular protein targeting and signal transduction.[1,13] The possibility to analyse its variation in healthy and diseased tissues is of great interest and will also help in elucidating the mechanisms by which methylation modifications of proteins exert control of diverse cell functions. The intensity of mRG expression is indeed quantitatively and qualitatively variable in normal tissues, as demonstrated by Western blot data and comparison of immunohistochemical staining across different tissues.

SPECIFICITY OF ANTIBODIES

The antibodies used for mRG detection and control staining reactions have been validated previously by enzyme-linked immunosorbent assay (ELISA), Western

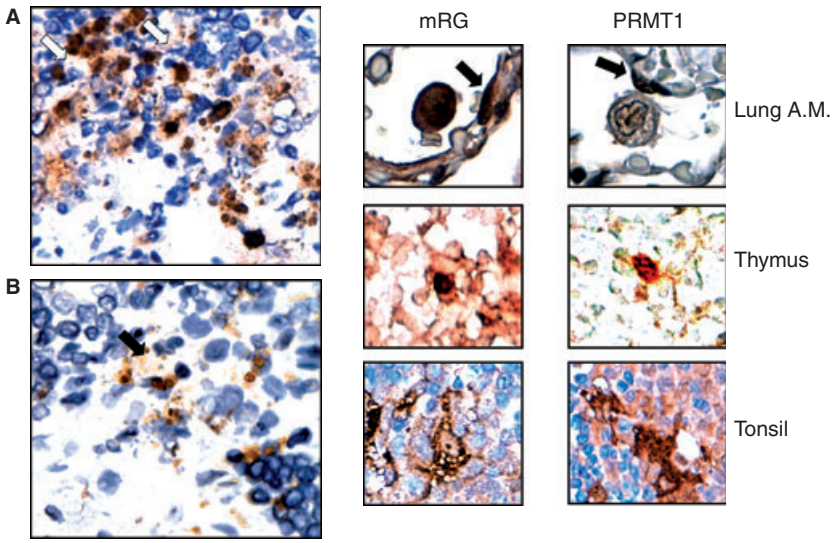

Figure 3. Methylarginine and protein arginine methyltransferase (PRMT1) expression in macrophages. Spleen stained with anti-methylarginine-specific antibody (anti-mRG) (**A**) and the control anti-RG antibody (**B**) Macrophages of the white pulp (white arrows in **A**) are stained by anti-mRG (**A**); note the presence of some tingible body macrophages of the white pulp (black arrow in **B**) characterized by brown pigment visible without a specific stain. On the right side are shown the myeloid cells present in the indicated tissues (AM: alveolar macrophage); the arrow in the lung indicates type 1 pneumocytes serving as internal positive control; note the lack of PRMT1 staining in alveolar macrophages in comparison with macrophages in the other tissues where both antigens are expressed.

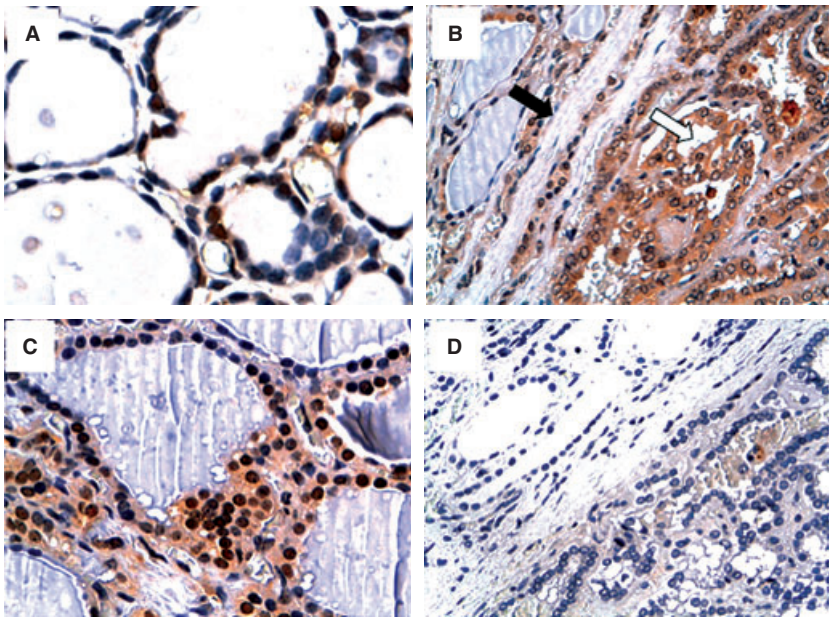

Figure 4. Methylarginine and protein arginine methyltransferase (PRMT1) expression in normal and neoplastic thyroid. Normal thyroid stained with anti-methylarginine-specific antibody (anti-mRG) (**A**) and anti-PRMT1 (**C**), showing nuclear and cytoplasmic staining for both antigens. Thyroid papillary carcinoma (white arrow) and adjacent non-neoplastic thyroid (black arrow) stained with anti-RG antibody show a predominant nuclear localization of mRG antigens in the latter, whereas the tumour exhibits mainly a cytoplasmic staining (**B**). Control anti-RG staining is shown in **D**.

blot and immunoprecipitation.[16] Application of either reagent to paraffin-embedded specimens of normal control tissue at the same immunoglobulin concentration yields dramatically different outcomes. Negligible to no staining is typical when using the control antibody. Staining alternate serial sections with anti-PRMT1, which recognizes the principal enzyme responsible for the generation of asymmetric dimethylarginine

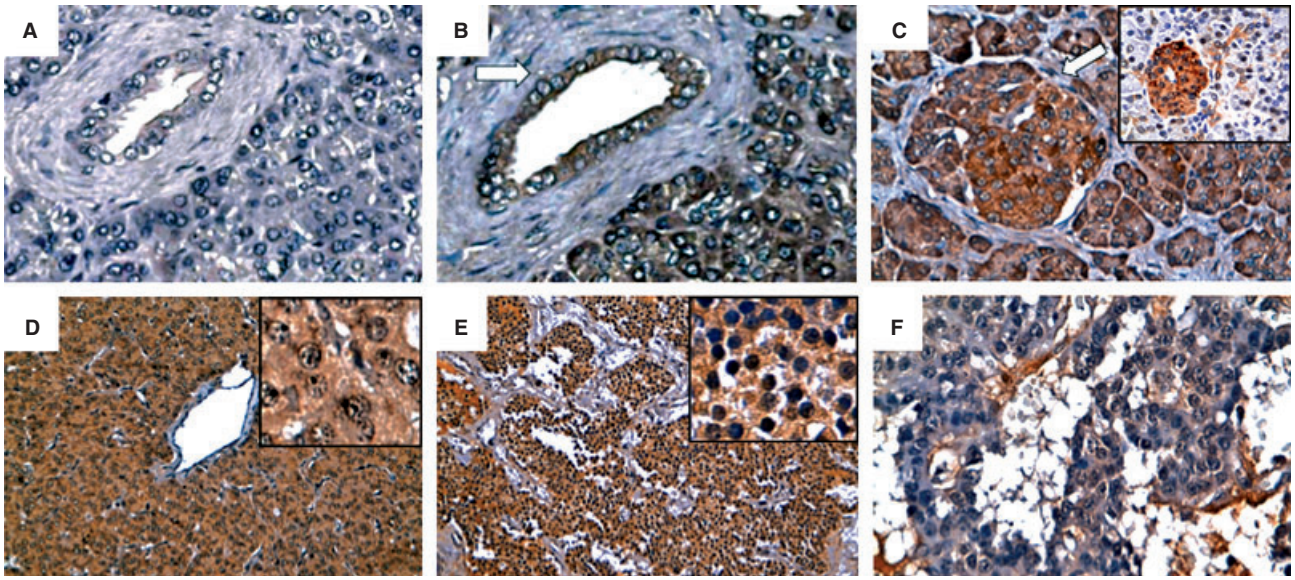

Figure 5. Methylarginine and protein arginine methyltransferase (PRMT1) expression in normal pancreas and in endocrine tumours. Upper panel: normal human pancreas stained with Control antibody (anti-RG) (**A**) and anti-mRG antibody (**B**, **C**). The white arrow indicates a ductule in **B** and a Langerhans's islet in **C**. Anti-PRMT1 staining is shown in the inset of **C**. Lower panel: three different pancreatic endocrine tumours showing a predominant cytoplasmic staining (**D**), mixed cytoplasmic and nuclear staining (**E**) or no staining (**F**).

Table 2. Expression of methylarginine-reactive protein (mRG) in various neoplasms

Cancer type	Cases	Positive	Negative	% Negative
Thyroid papillary	8	8	0	0
Breast	64	60	4	4.6
Colon	19	15	4	22
Gastric	32	25	7	21
Pancreatic ductal	25	17	8	32
Pancreatic endocrine	10	8	2	20

in eukaryotic cells, provides further independent and complementary assessment of antibody specificity. Using this approach we demonstrate, for example, that syncytial trophoblasts are devoid of mRG signal and do not express PRMT1. In contrast, the decidual cells are strongly positive for both antigens. The correlation of staining intensity for methylarginine and PRMT1 was demonstrated for nearly all the other tissues analysed and supports the conclusion that the anti-mRG antibody specifically detects asymmetric dimethylarginine substrates in the same cellular compartment as PRMT1. Few exceptions to this rule were observed; namely, in thymocytes and alveolar macrophages, there was strong mRG staining, but no expression of PRMT1. In different nearby cells PRMT1 was, however, present, thus excluding the occurrence of artefacts. In such cases we suspect that enzymes other than PRMT1 catalyse the addition of mRG to cell proteins, suggesting the activation of specific signalling pathways in these cell types.

SENSITIVITY OF ANTIBODIES

The sensitivity of the mRG detection illustrated in the data images in this report was quite favourable, as suggested by a low tissue background and a resultant high signal-to-noise in all tissues examined. Further evidence of excellent sensitivity can be inferred by a recent study on the functional domains of the *Schizosaccharomyces pombe* Rmt3 arginine methyltransferase. Using anti-mRG to assess the ability of Rmt3 variants to methylate ribosomal protein S2 (Rps2), the authors found a relative quantitative correlation between anti-mRG staining of native Rps2 methylated by the Rmt3 variants and *in vitro* radioactive methylation of Rps2 by the same series of recombinant Rmt3 variants.[18]

OBSERVATIONS ON NORMAL TISSUES

MeRP detectable with anti-mRG antibody were distributed widely but not ubiquitously. For example, high expression was detected in germinal centres while the surrounding lymphocytes are negative. This indicates

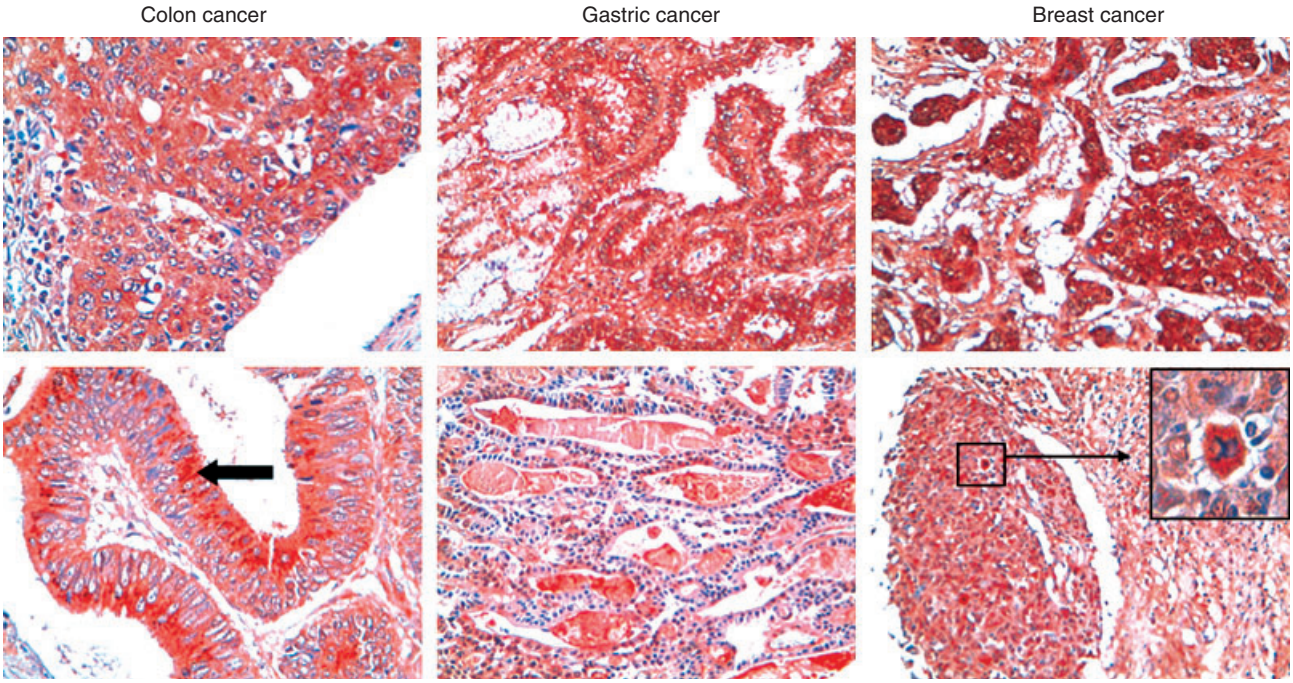

Figure 6. Distinctive patterns and levels of methylarginine protein are detected in colonic, gastric and breast cancers. Tumour samples stained with anti-methylarginine-specific antibody (anti-mRG). Examples of a colonic cancer showing cytoplasmic mRG expression (upper panel) and of a colonic cancer with perinuclear/Golgi-like signal (lower panel). Examples of gastric and breast cancers with high mRG cytoplasmic expression are shown in the upper panel, and cases with a faint signal in the lower panels. The inset shown in the lower right image (breast cancer) displays a mitosis where mRG signal is clearly higher than that of the surrounding cells.

that B cell differentiation/proliferation is associated with the increase of mRG levels. This situation is reminiscent of the role of arginine methylation in the epigenetic transcription events associated with myeloid cell differentiation.[19] The differences in subcellular distribution, along with probable differences of methylation reactions in different normal tissues, will need to be addressed by future studies in order to understand better the possible physiopathological relevance of these findings. An initial step towards this direction was the comparative analysis among normal and neoplastic cells derived from the same tissues presented here and discussed in the following section.

OBSERVATIONS ON NEOPLASIA

The distribution of mRG expression in carcinoma was of particular interest, given recent observations regarding the involvement of PRMTs in genome stability,[20] DNA repair[21] and oncogenesis.[22] The discovery that methylation of p53 is required for the regulation of its activity[3,23] highlights further the potential importance of the signalling pathways regulated by this post-translational modification in tumours. The potential use of mRG immunohistochemistry for the study of alterations in protein arginine methylation in primary tumours was suggested by the differential staining observed between normal and neoplastic tissues. Moreover, and at variance with the matched normal tissue, cancer showed heterogeneous mRG staining characteristics, both quantitative and qualitative, including perinuclear Golgi-like staining or different nuclear-to-cytosol ratio, hyper- or hypoexpression (Figures 4–6). Several possible molecular alterations that may occur as a consequence of tumorigenesis could give rise to the heterogeneity of tumour staining. Hypo- or, perhaps, hypermethylation of constitutive protein substrates may lead to aberrant targeting of the methylprotein.[24] Variant alternatively spliced forms of PRMT1[9] may also influence the staining characteristics of mRG and, lastly, differential effects of putative protein arginine demethylases[25] may play some role in mRG staining.

Considered together, our results imply that mRG levels are regulated differentially in different tumour types and might thus play various roles in neoplastic transformation, according to the pathways specific for individual tumour histotypes. This speculation is supported by the observation that gastrointestinal tract and pancreatic endocrine tumours share a similar level of mRG downregulation (between 20 and 30%), at variance with breast (4.6%) and thyroid papillary cancers (0%).

Finally, it is important to recognize the limitations of the method that we describe. Because the immunogen used was a repetitive methylation motif common to the majority of methylarginine proteins, a large number of reactive methylarginine proteins could be expected to react with the antibody. We have not determined formally whether the antibody recognizes only asymmetric dimethylarginine or monomethylarginine, and also symmetric dimethylarginine. We could also not exclude the remote possibility that the antibody will react with free asymmetric dimethylarginine that is not part of a protein amide-bonded backbone. The issues of methyl-type specificity will be addressed by future biochemical studies.

In conclusion, this study demonstrated that anti-methylarginine and anti-PRMT1 antibodies can detect specifically the respective antigens in paraffin-embedded tissues and can reveal alteration in the expression/processing of mRG. The procedure can be utilized for the study of archival material and the screening of large collections of normal and diseased tissues and, thus, contribute to a greater understanding of the pathophysiological role of arginine methylation.

Competing interests

J.M.A. is a cofounder and the Principal Scientist of CH3 BioSystems LLC. He is named as inventor on USPTO 6699673, 'Protein Methylarginine-Specific Antibodies' and on the provisional patent application 'Methylarginine Detection in Cells and Tissues'.

Acknowledgements

This study was supported by Associazione Italiana Ricerca sul Cancro (AIRC) Veneto Region Grant, Milan, Italy; Fondazione Cariparo, Padova, Italy; Ricerca Sanitaria Finalizzata Regione Veneto; and Ministero della Salute – project RF-EMR-2006-361866; European Community FP6 'MolDiagPaca'.

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