CELL DIFFERENTIATION AND MORPHOLOGIC HETEROGENEITY OF GASTRIC CARCINOMA. INSIGHTS FROM THE IMMUNOCYTOCHEMICAL AND MOLECULAR STUDY OF TREFOIL PEPTIDES AND E-CADHERIN.

DIFERENCIAÇÃO CELULAR E HETEROGENEIDADE MORFOLÓGICA DO CARCINOMA GÁSTRICO. ESTUDO IMUNOCITOQUÍMICO E MOLECULAR DE "TREFOIL PEPTIDES" E DA CADERINA-E.

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DISSERTAÇÃO DE CANDIDATURA AO GRAU DE DOUTOR APRESENTADA À FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO.

Artigo 48°, § 3° – A Faculdade não responde pelas doutrinas expendidas na dissertação. (Regulamento da Faculdade de Medicina do Porto – Decreto-Lei n° 19337, 29 de Janeiro de 1931).

Ao abrigo do Art. 8 ° do Decreto-Lei nº 388/70 fazem parte integrante desta dissertação os seguintes trabalhos já publicados ou em publicação:

JC Machado, F Carneiro, N Blin, M Sobrinho-Simões. Pattern of pS2 protein expression in premalignant and malignant lesions of gastric mucosa. *European Journal of Cancer Prevention* 1996, **5**: 169-179.

JC Machado, F Carneiro, P Ribeiro, N Blin, M Sobrinho-Simões. pS2 protein expression in gastric carcinoma. An immunohistochemical and immunoradiometric study. *European Journal of Cancer* 1996, **32A**: 1585-1590.

AMMF Nogueira, JC Machado, F Carneiro, CA Reis, P Gött, M Sobrinho-Simões. Patterns of expression of trefoil peptides and mucins in gastric polyps with and without malignant transformation. *Journal of Pathology* 1999, **187**: 541-548.

JC Machado, AMMF Nogueira, F Carneiro, CA Reis, M Sobrinho-Simões. Gastric carcinoma exhibits distinct types of cell differentiation: An immunohistochemical study of trefoil peptides (TFF1 and TFF2) and mucins (MUC1, MUC2, MUC5AC and MUC6). *Journal of Pathology* (submetido para publicação).

JC Machado, F Carneiro, S Beck, S Rossi, J Lopes, A Taveira-Gomes, M Cardoso-Oliveira. E-cadherin expression is correlated with the isolated cell/diffuse histotype and with features of biological aggressiveness of gastric carcinoma. *International Journal of Surgical Pathology* 1998, **6**: 135-144.

JC Machado, P Soares, F Carneiro, A Rocha, S Beck, N Blin, G Berx, M Sobrinho-Simões. E-cadherin gene mutations provide a genetic basis for the phenotypic divergence of mixed gastric carcinomas. *Laboratory Investigation* (em publicação).

Em cumprimento do disposto no referido Decreto-Lei declara que participou activamente na recolha e estudo do material incluído em todos os trabalhos, tendo redigido os textos com a colaboração dos outros autores.

NOTA EXPLICATIVA

A presente dissertação está escrita em inglês na sua quase totalidade, exceptuando o Sumário e Conclusões, pelo facto de o Professor Nikolaus Blin ter sido o seu orientador.

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INTRODUCTION

Foreword

According to the last estimates, the incidence of gastric carcinoma is the second in rank world-wide (Parkin, 1998). In the so-called developed western countries, a steady decrease in the incidence of gastric carcinoma has been observed during the last two to three decades (Howson, *et al.* 1986; La Vecchia, *et al.* 1992). In contrast, this tendency is not so clear in Portugal, where the incidence and mortality rates of gastric carcinoma are among the highest in the world (Parkin, *et al.* 1988) and are even the highest in the European Union (Black, *et al.* 1997). In Portugal, gastric carcinoma is the second most incident cancer and the major cause of cancer mortality (Parkin, 1998).

In a very simplified way, it is generally accepted that there are two main pathways of malignant transformation of gastric mucosa: one *via* intestinal metaplasia and adenomatous dysplasia, leading to intestinal carcinomas; the other *via* hyperplastic or *de novo* changes, with or without concurrent non-metaplastic dysplasia, leading to diffuse carcinomas and to a subset of intestinal carcinomas (Correa, 1992; Solcia, *et al.* 1996; Carneiro, 1997).

Morphologic diversity of gastric carcinoma is much greater than what could be anticipated by the recognition of two main variants of carcinoma arising in the stomach — intestinal and diffuse. In fact, a large proportion of gastric carcinomas are polymorphic exhibiting a large diversity both at structural and cell differentiation level and the molecular counterpart underlying this heterogeneity is not well elucidated.

Among the plethora of candidate molecular factors, potentially involved in the pathogenesis of gastric carcinoma, trefoil peptides and the cell adhesion molecule E-Cadherin have been chosen, in the present study, as tools to analyse some aspects of cell differentiation and morphologic heterogeneity of gastric carcinoma.

Broadly, trefoil peptides and E-Cadherin are involved in fundamental biological processes such as cell adhesion and motility and are normal constituents of the human gastric epithelium. Data on record support that disturbed function of these molecules

may play an important role in gastric carcinogenesis and in the biopathology of these tumours.

In the following pages of the introductory section of this Thesis, a general overview on the morphologic heterogeneity and cell differentiation of gastric carcinoma, as well as on its putative molecular counterparts, will be presented. Furthermore, emphasis will be given to the general characterisation of the trefoil peptides and E-Cadherin molecules and to their potential role in cancer development.

Classification, differentiation and ethiopathogenesis of gastric carcinoma

From the morphologic standpoint gastric carcinoma is very heterogeneous, probably reflecting the complexity and diversity of the process(es) of malignant transformation of gastric mucosa (Laurén, 1965; Correa, 1992; Carneiro, *et al.* 1995b). This heterogeneity is amply reflected in the diversity of histopathological classifications on record (Borrman, 1926; Laurén, 1965; Ming, 1977; Watanabe, *et al.* 1991; Carneiro, *et al.* 1995b), which are based on different criteria such as histological profile, degree of differentiation, histogenesis and pattern of growth.

According to Laurén's classification (1965), there are two main types of gastric carcinoma — intestinal and diffuse carcinoma — which differ from each other morphologically, clinically and epidemiologically. Using Laurén's classification as a framework, Carneiro *et al* (1995b) proposed a histopathological classification of gastric carcinoma based on the tissue organisation of the tumours and individualising the group of cases designated as mixed carcinomas that display, in the same tumour a glandular/solid component and an isolated-cell component. The four groups of the proposed classification, found to carry independent prognostic meaning, are glandular carcinoma, isolated-cell carcinoma, solid carcinoma and mixed carcinoma. Glandular and isolated-cell carcinomas correspond to carcinomas that, according to Laurén's classification, are designated as intestinal and diffuse carcinomas, respectively. In our opinion, these two classifications are those that allow a more adequate interpretation of both the epidemiological and the ethiopathogenic aspects of gastric carcinoma. Accordingly, they will be used throughout the studies that constitute this Thesis.

Morphologically, the diffuse carcinoma (Fig. 1A) is characterised by displaying isolated tumour cells, scattered throughout the stroma, which infiltrate the surrounding tissues and are associated with a desmoplastic reaction. The intestinal carcinoma (Fig. 1B) is characterised by the formation of glandular structures with an expansive type of growth.

At the clinicopathological level, the two main types of gastric carcinoma also show different behaviour: the diffuse carcinomas tend to invade locally the surrounding

tissues, to disseminate to the peritoneum and, through the lymphatic vessels, to the regional lymph nodes; intestinal carcinomas characteristically give rise to blood born metastasis (Esaki, *et al.* 1990).

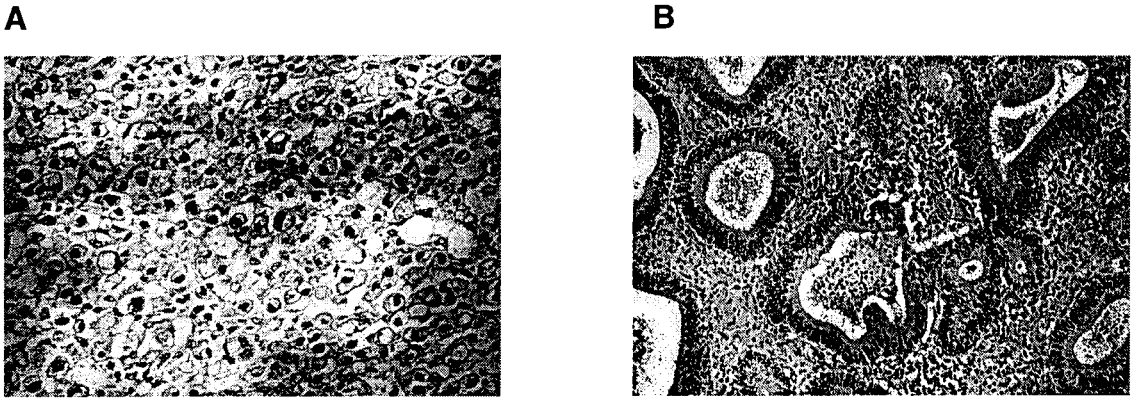


Fig. 1 – Typical examples of a diffuse carcinoma (A) and of an intestinal carcinoma (B).

From the epidemiological standpoint, diffuse carcinomas occur more frequently in young individuals and mainly in women, whereas intestinal carcinomas occur often in elderly individuals and more frequently in men (Ribeiro, *et al.* 1981; Mecklin, *et al.* 1988; Maehara, *et al.* 1992). Earlier studies pointed to a hereditary conditioning of diffuse carcinomas occurring in the setting of familial gastric cancer (Lehtola, 1978; Mecklin, *et al.* 1988). More recent reports have described similar proportions of diffuse and intestinal gastric cancer in patients with a positive familial history (Palli, *et al.* 1994; Shinmura, *et al.* 1998).

While the term "diffuse" is mainly related with the pattern of growth of this specific type of gastric carcinoma, the term "intestinal" encompasses two different concepts; the first is architectural and points to a tumour with a structure similar to that of most colonic carcinomas, while the second is histogenetic and based on the assumption that this type of gastric carcinoma originates from intestinal metaplasia of the stomach. Intestinal-type differentiation was described several years ago in gastric cancer (Laurén, 1965; Goldman and Ming, 1968a; Goldman and Ming, 1968b) and it was believed that

this type of metaplastic differentiation was typical of intestinal-type gastric carcinomas (Laurén, 1965; Ghandur-Mnaymneh, *et al.* 1988).

In recent years, however, ultrastructural studies (Fiocca, *et al.* 1987; Fiocca, *et al.* 1990; Carneiro, *et al.* 1992) showed that differentiated cell types of both gastric type (foveolar and mucopeptic) and intestinal type (columnar and goblet cells) can be recognised in most gastric tumours, regardless of the histological type. Moreover, histochemical and immunohistochemical studies (Bara, *et al.* 1981; Tatematsu, *et al.* 1986; Fiocca, *et al.* 1987; Fiocca, *et al.* 1988a; Fiocca, *et al.* 1988b; Fiocca, *et al.* 1990; Kushima and Hattori, 1993; Kushima, *et al.* 1993), aiming at the identification of gastric-type and intestinal-type antigens in gastric carcinoma, provided additional support to the aforementioned ultrastructural findings. These results showed that both types of cell differentiation (gastric and intestinal) can be observed, alone or in combination, in intestinal as well as in diffuse carcinomas. Table 1 summarises the ultrastructural, histochemical and immunohistochemical features that serve to identify gastric- and intestinal-type cell differentiation.

Correa (1988; 1992) proposed a model of gastric carcinogenesis according to which the intestinal carcinoma represents the end-product of a cascade of sequential changes of gastric mucosa including superficial gastritis, chronic atrophic gastritis, small intestinal metaplasia, colonic metaplasia and adenomatous (flat or polypoid) dysplasia. According to this model the major etiological factors would be excessive salt intake, *Helicobacter pylori* infection, low intake of ascorbic acid and carotenoids and intragastric nitrosation due to nitrate intake (Correa, 1988; Correa, 1992). However, Correa's model does not fit with the subset of intestinal carcinomas that show histochemical signs of gastric-type differentiation and no evidence of intestinal-type differentiation (Fiocca, *et al.* 1987; Fiocca, *et al.* 1988a; Carneiro, *et al.* 1992; Kushima and Hattori, 1993).

Table 1 – Ultrastructural, histochemical and immunohistochemical "markers" used to identify gastric- and intestinal-type differentiation in gastric carcinoma.

Cell differentiation	"Markers"
Gastric-type	Punctate cerebroid granules[1]
	Target-like or solid granules[2]
	GOS reactive mucins[1]
	PACONA reactive mucins[2]
	Cathepsin-E[1]
	Pepsinogen II[2]
	Neutral mucins[1,2]
	M1 and M2[1]
Intestinal-type	Microvilli with cytoplasmic roots[3]
	Clear, coalescing granules[4]
	CAR-5[3,4]
	Sialyl-Tn[3,4]
	Acid mucins[3,4]
	BD-5[3,4]
	M3[4]

Markers of gastric foveolar cells (1), gastric mucopeptic cells (2), intestinal columnar cells (3) and intestinal goblet cells (4); GOS – Galactose-oxidase Schiff stain; PACONA – Periodic-acid-biotynilated concanavalin A stain. For a review see refs. (Bara, *et al.* 1981; Tatematsu, *et al.* 1986; Fiocca, *et al.* 1987; Fiocca, *et al.* 1988a; Fiocca, *et al.* 1988b; Fiocca, *et al.* 1990; Carneiro, *et al.* 1992; Kushima and Hattori, 1993; Kushima, *et al.* 1993).

From the histogenetic standpoint it is generally accepted that diffuse carcinomas originate from gastric proper mucosa and that non-metaplastic dysplasia is a putative premalignant lesion of this type of carcinoma (Ghandur-Mnaymneh, *et al.* 1988), though little is known regarding the prevalence and clinicopathological features of the aforementioned type of dysplasia. Furthermore, there is growing evidence suggesting that hyperplastic lesions of gastric mucosa (foveolar hyperplasia and hyperplastic polyps) may also serve as precursor lesions of gastric carcinoma, namely of diffuse-type (Carneiro, *et al.* 1993a; Carneiro, *et al.* 1993b; Zea-Iriarte, *et al.* 1995).

In summary, the aforementioned evidence suggests the existence of at least two pathways of malignant transformation of gastric mucosa: one *via* intestinal metaplasia and adenomatous (flat or polypoid) dysplasia, leading to intestinal carcinomas with

intestinal-type differentiation; the other *via* hyperplastic or *de novo* changes, with or without concurrent non-metaplastic dysplasia, leading to diffuse carcinomas and to a subset of intestinal carcinomas with gastric-type differentiation (Carneiro, 1997) (Fig. 2).

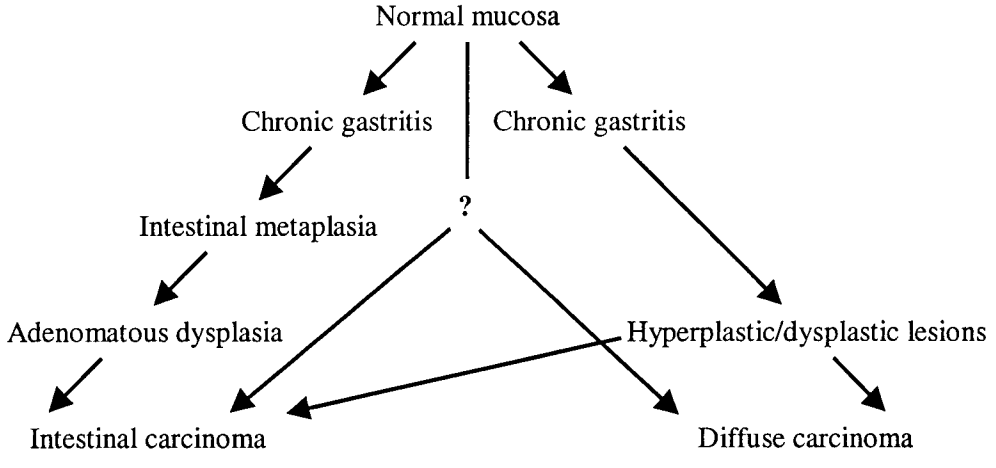


Fig. 2 – Model of gastric carcinogenesis illustrating the two main histogenetic pathways.

Molecular counterpart of the morphologic heterogeneity of gastric carcinoma

Cancer has long been recognised as a multistage process involving, in most cases, an accumulation of multiple genetic alterations as the result of successive rounds of mutation and clonal selection in tumour cells (Nowell, 1976; Knudson, Jr. 1985), and the establishment of a specific "environment" in the tumour which involves an altered balance of transcription factors and expression/repression of different genes (Tahara, 1990; Brattain, *et al.* 1994). Although the precise nature and sequence of those alterations remain mostly unclear, several abnormalities affecting both structure and expression of a vast number of genes have been identified (Fearon and Vogelstein, 1990; Levine, 1990; Vogelstein and Kinzler, 1993; Tahara, 1995; Hussain and Harris, 1998; Lengauer, *et al.* 1998). We are now aware that most of these genes code for proteins that belong to functional groups such as growth factors and their receptors, cell cycle regulators, hormone receptors, cell adhesion molecules, signal transduction proteins, DNA repair proteins, transcription factors or apoptosis related proteins.

However, despite the huge amount of information regarding molecular changes that occur in gastric carcinoma and that have been identified by many groups using different methods, a sound molecular model for gastric carcinogenesis is far from being established (Lemoine, *et al.* 1992; Seruca, *et al.* 1992; Wright and Williams, 1993; Carneiro, *et al.* 1994; Correa and Shiao, 1994; David, *et al.* 1994; Seruca, *et al.* 1995; Santos, *et al.* 1996; Tahara, 1995; Amado, *et al.* 1998).

Table 2 summarises, in a very simplified way, the most prominent data on the molecular alterations of gastric carcinoma, according to the two major histological types (diffuse and intestinal). Table 2 includes data on DNA content, loss of heterozygosity (LOH) in several chromosomes, microsatellite instability (MSI), proto-oncogenes (*KSAM*, *RAS*, *ERBB2* and *MET*), tumour suppressor genes (*P53*, *APC* and *DCC*), growth factors (EGF, TGF-α and TGF-β), glycoconjugates (S-Tn, T, S-T, CDw75 and S-Le^x antigens), matrix components (laminin and collagen IV) and degradating matrix enzymes (u-Pa and Cat-D).

Table 2 – Summary of the molecular alterations in gastric carcinoma according to the two main histological types.

Intestinal carcinoma	**Diffuse carcinoma**
Aneuploidy	Diploidy
# 3p,6q,7q,13q (LOH)	# 3p,6q,7q,13q (LOH)
# 1q,5q,17p (LOH)	
MSI+	
RAS (mutation/overexpression)	
ERBB2 (amplification)	
TPR-MET rearrangement	
MET (6.0 Kb mRNA)	*MET* (6.0 Kb mRNA)
	KSAM amplification
P53 (LOH, mutation)	*P53* (LOH, mutation)
APC (LOH, mutation)	
DCC (LOH)	
EGF overexpression	
TGF-α overexpression	
	TGF-β overexpression
S-Tn, T, S-T	S-Tn, T, S-T
CDw75, S-Le^x	CDw75, S-Le^x
Laminin, collagen IV	
u-Pa, Cat-D	

For a thorough review see refs. (Lemoine, *et al.* 1992; Seruca, *et al.* 1992; Wright and Williams, 1993; Carneiro, *et al.* 1994; Correa and Shiao, 1994; David, *et al.* 1994; Seruca, *et al.* 1995; Santos, *et al.* 1996; Tahara, 1995; Machado, *et al.* 1997; Amado, *et al.* 1998).

Taking together all the above evidence it is possible to conclude that some of the molecular alterations occur exclusively, or predominantly, in each of the major histotypes of gastric carcinoma; for example, aneuploidy, 5q and 17p LOH, MIN+ phenotype, *ERBB2* amplification, EGF and TGF-α overexpression are found mainly in intestinal carcinomas; at variance, diploidy, *KSAM* amplification and TGF-β overexpression are associated with diffuse carcinoma. Although a clear understanding of the molecular pathogenesis of gastric carcinoma is at an early stage, these observations support the assumption that there is, indeed, a molecular counterpart

underlying the two major histotypes of gastric carcinoma, which is in keeping with the existence of at least two histogenetic pathways of gastric carcinogenesis.

However, this model is rendered more complex by the existence of mixed gastric carcinomas, that present both a diffuse histotype component and an intestinal histological component. It remains to be seen if mixed gastric carcinomas share molecular features of the "pure" histological types or if, on the contrary, they exhibit a distinct molecular profile.

Trefoil peptides

General features

Trefoil peptides are a group of small stable secreted peptides characterised by the presence of one up to six cysteine-rich domains, designated as trefoil domains (formerly P-domains) (Thim, 1989; Hoffmann and Hauser, 1993). This basic module defining the trefoil peptide family consists of 38 or 39 amino acids ($CX_9CX_9CX_4CCX_9WCF$) folded into a clover-leaf structure, and held together by three disulphide bridges (Fig. 3) (Thim, 1989; Podolsky, *et al.* 1993).

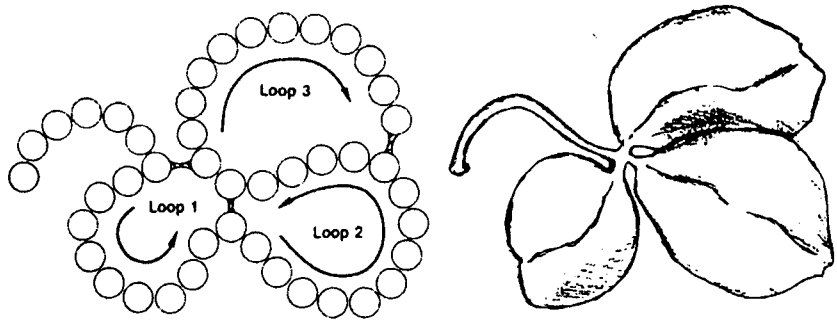


Fig. 3 – Analogy between the primary structure of a trefoil domain and a trefoil leaf, with the disulphide bonding of cysteine residues 1-5, 2-4 and 3-6. Adapted after Thim (1994).

Members of this family of peptides have been discovered in different species from *Xenopus laevis* to human (Jorgensen, *et al.* 1982b; Masiakowski, *et al.* 1982; Hoffmann, 1988; Tomasetto, *et al.* 1990; Hauser and Hoffmann, 1991; Suemori, *et al.* 1991; Hauser, *et al.* 1992; Hauser and Hoffmann, 1992; Hauser, *et al.* 1993; Podolsky, *et al.* 1993). Table 3 summarises data regarding the number of trefoil domains and main sites of expression of known trefoil peptides. Interestingly, regardless of species and of organ of expression, all trefoil peptides are expressed in mucin secreting epithelia.

Table 3 – Number of trefoil domains and major sites of expression of trefoil peptides grouped according to their homology.

Name	Trefoil domains	Typical localisation	References
human TFF1	1	Stomach	(Masiakowski, *et al.* 1982; Rio, *et al.* 1988)
mouse TFF1	1	Stomach	(Otto, *et al.* 1996)
X. laevis xP1	1	Stomach	(Hauser and Hoffmann, 1991)
human TFF2	2	Stomach	(Tomasetto, *et al.* 1990)
mouse TFF2	2	Stomach	(Tomasetto, *et al.* 1990)
porcine TFF2	2	Pancreas	(Rose, *et al.* 1989; Tomasetto, *et al.* 1990)
human TFF3	1	Intestine	(Hauser, *et al.* 1993; Podolsky, *et al.* 1993)
rat TFF3	1	Intestine	(Suemori, *et al.* 1991; Chinery, *et al.* 1992)
X. laevis xP2	2	Skin	(Hauser, *et al.* 1992)
X. laevis xP4	4	Stomach	(Hauser and Hoffmann, 1991)
X. laevis FIM-A.1	4	Skin	(Hoffmann, 1988; Hauser, *et al.* 1990)
X. laevis FIM-C.1	6	Skin	(Hauser and Hoffmann, 1992)

FIM – Frog integumentary mucin

Three human trefoil peptides have been identified to date: TFF1 (formerly pS2) and TFF3 (formerly hITF) with one trefoil domain each, and TFF2 (formerly hSP) with two trefoil domains. TFF1 was originally identified in 1982 as the product of an oestrogen-responsive gene in the breast carcinoma cell line MCF-7 (Masiakowski, *et al.* 1982); TFF2 was discovered in 1990 (Tomasetto, *et al.* 1990) as a human homologue of a peptide isolated from porcine pancreas, the porcine TFF2 (formerly PSP) (Rose, *et al.* 1989); and TFF3 was identified in 1993 (Hauser, *et al.* 1993; Podolsky, *et al.* 1993) as a human homologue of rat TFF3 (Suemori, *et al.* 1991).

Expression of trefoil peptides in normal tissues

In normal human tissues, trefoil peptides are mainly expressed in gastrointestinal epithelial cells, where they are copackaged by the Golgi apparatus into mucus granules and secreted with mucins into the protective layer covering the mucosa (Hanby, *et al.* 1993b; Sarraf, *et al.* 1995; Poulsom, 1996). Table 4 summarises data regarding the sites of physiological expression of trefoil peptides in normal human tissues.

Table 4 – Sites of expression of trefoil peptides in normal human tissues.

Trefoil peptide	Organ	Description	References
TFF1	Stomach	Throughout superficial and foveolar mucosa	(Rio, *et al.* 1988; Luqmani, *et al.* 1989; Hanby, *et al.* 1993b)
	Duodenum	Upper ducts and surface cells of Brunner's glands	(Hanby, *et al.* 1993a)
	Large intestine	Some goblet cells of distal regions	(Singh, *et al.* 1998)
	Pancreas	Focally in duct epithelium	(Wright, *et al.* 1990)
	Gall bladder	Patchy epithelial expression	(Seitz, *et al.* 1991)
	Salivary glands	Patchy expression	(Rio, *et al.* 1988)
	Breast	Focally in duct luminal cells	(Poulsom, *et al.* 1997)
TFF2	Stomach	Mucous glands of body and antrum	(Hanby, *et al.* 1993b)
	Duodenum	Brunner's glands acini and distal ducts	(Piggott, *et al.* 1991)
	Pancreas	Focally in duct epithelium	(Wright, *et al.* 1990)
	Gall bladder	Patchy epithelial expression	(Seitz, *et al.* 1991)
TFF3	Duodenum	Brunner's glands acini and ducts, goblet cells	(Suemori, *et al.* 1991; Chinery, *et al.* 1992; Hauser, *et al.* 1993; Podolsky, *et al.* 1993)
	Small and large intestine	Goblet cells	(Suemori, *et al.* 1991; Chinery, *et al.* 1992; Hauser, *et al.* 1993)
	Uterus	Epithelium	(Hauser, *et al.* 1993)
	Breast	Focally in duct luminal cells	(Poulsom, *et al.* 1997)
	Brain	Hypothalamus, pituitary	(Probst, *et al.* 1995; Schwarzberg, *et al.* 1999)

The main site of expression of TFF1 is the stomach, where it is abundantly expressed in the superficial and foveolar epithelium (Rio, *et al.* 1988; Luqmani, *et al.* 1989; Hanby, *et al.* 1993b). A high level of expression of TFF1 has also been described in upper ducts

and surface cells of Brunner's glands in the duodenum (Hanby, *et al.* 1993a). The small intestine generally appears not to express TFF1, although some staining of the tips of villi in the ileum and jejunum has been reported (Piggott, *et al.* 1991). In the normal large intestine, TFF1 expression has been demonstrated in some goblet cells, particularly in the distal regions (Singh, *et al.* 1998). In the pancreas only a few cells in large ducts appear positive (Wright, *et al.* 1990) and in gall bladder some patchy epithelial expression has been described (Seitz, *et al.* 1991). Salivary glands were reported as weakly immunopositive (Rio, *et al.* 1988). Outside of the gastrointestinal tract, TFF1 expression has been observed focally in duct luminal cells of normal breast (Poulsom, *et al.* 1997) (Table 4).

The expression of TFF2 appears to be highly correlated with that of TFF1 in terms of organ specificity. In normal gastric mucosa, TFF2 expression is observed in mucous glands of body and antrum (Hanby, *et al.* 1993b). In duodenum, TFF2 expression is present in Brunner's glands acini and distal ducts (Piggott, *et al.* 1991). Some focal expression is also observed in duct epithelium of pancreas (Wright, *et al.* 1990) and in gall bladder epithelium (Seitz, *et al.* 1991). In contrast with TFF1, which shows a good agreement between data on protein and mRNA expression, some discrepancies have been reported for TFF2; in gastric mucosa TFF2 mRNA has been detected in the glands and in the foveolar epithelium, but the peptide is undetectable in the surface cells (Hanby, *et al.* 1993b) (Table 4).

While TFF1 and TFF2 are mainly expressed in the stomach, the major site of expression for TFF3 is the intestine. TFF3 is expressed in goblet cells throughout the intestine and in gland acini and distal ducts of Brunner's glands (Suemori, *et al.* 1991; Chinery, *et al.* 1992; Hauser, *et al.* 1993; Podolsky, *et al.* 1993). In contrast with the apparent gastrointestinal specificity of TFF1 and TFF2, TFF3 expression has been observed in human uterus (Hauser, *et al.* 1993), normal breast (Poulsom, *et al.* 1997), some regions of the hypothalamus and in the pituitary gland (Probst, *et al.* 1995; Schwarzberg, *et al.* 1999) (Table 4).

Structure of trefoil peptide genes and proteins

The TFF1 locus, known as breast cancer oestrogen inducible (*BCEI*), is composed of three exons (Rio and Chambon, 1990); the single trefoil domain of TFF1 is coded by exon 2. The 5'-promoter region of the *BCEI* gene contains an oestrogen responsive element (ERE) as well as elements responsive to phorbol esters, c-Ha-*ras* and c-*jun* (Berry, *et al.* 1989; Nunez, *et al.* 1989). The gene structure of the TFF2 gene, whose locus is denominated *SML1* (spasmolysin 1), exhibits four exons, two of which encode the two separate trefoil domains (Tomasetto, *et al.* 1990). Analysis of the human *TFF3* gene reveals the same basic structure of three exons as human *BCEI* (Hauser, *et al.* 1993; Podolsky, *et al.* 1993).

The three human trefoil peptide genes are clustered in the genomic region 21q22.3 (Theisinger, *et al.* 1992; Tomasetto, *et al.* 1992; Chinery, *et al.* 1996; Gött, *et al.* 1996; Schmitt, *et al.* 1996), in a tandemly oriented fashion and within a 50 kbp genomic fragment (Gött, *et al.* 1996). The locus order is cen-*TFF3-SML1-BCEI*-tel and the transcription of all three genes is directed towards the centromere (Gött, *et al.* 1996).

Analysis of the exon structure of the three trefoil peptide genes reveals a very similar organisation among them. The first exon encodes the secretion signal sequence, the second exon (and the third exon of *SML1*) encode the trefoil domains, and the third exon (fourth exon of *SML1*) encodes three to four residues of the carboxyl terminus (Gött, *et al.* 1996). This conserved structural organisation may have evolved by gene duplication and exon shuffling (Gött, *et al.* 1996).

Besides gene clustering and an identical transcription orientation of all three trefoil peptide genes, the 5'-flanking regions share several motifs with almost identical sequences and spacing, suggesting a co-ordinated regulation and/or a common locus-controlling region (Gött, *et al.* 1996). Moreover, two motifs with identical sequence and position are shared exclusively by the stomach-specific genes *BCEI* and *SML1*, thus presenting possible targets for stomach specific gene regulation (Gött, *et al.* 1996).

The three human members of the trefoil peptide family are small and stable secreted proteins: TFF1 is 84 amino acids long in its immature form and 60 amino acids long in

its mature form (Mori, *et al.* 1988); the immature TFF2 is 129 amino acids long and its mature form is 106 amino acids long (Tomasetto, *et al.* 1990); TFF3 has a sequence of 80 amino acids in the immature form and 59 amino acids in the mature form (Thim, *et al.* 1995).

The characteristic trefoil domain of these peptides presents a compact tri-dimensional trefoil structure that appears to confer marked resistance to proteolysis and acid digestion (Jorgensen, *et al.* 1982b; Mori, *et al.* 1988; Playford, *et al.* 1995; Thim, *et al.* 1995).

There is some evidence that trefoil peptides can exist naturally as dimers; TFF1 can bridge through the seventh cysteine residue either to another TFF1 molecule (Chadwick, *et al.* 1995; Chinery, *et al.* 1995) or perhaps to TFF3 (Chinery, *et al.* 1995); likewise, for TFF3 the possibility of homo-dimerisation was also demonstrated (Chinery, *et al.* 1995; Thim, *et al.* 1995). These data show that dimerisation is essential for some biological properties of trefoil peptides and raise the intriguing possibility that a spectrum of biological activities may be generated by combining different single-trefoil peptides.

Function of trefoil peptides

The first studies on the biological activity of trefoil peptides were performed by Jorgensen *et al* (1982a) on porcine TFF2 in experimental animals. These studies suggested that these peptides had a role in inhibition of gastrointestinal motility and in inhibition of pentagastrin-induced acid secretion (Jorgensen, *et al.* 1982a). However, recent studies on the human and porcine peptides have not supported these findings (Playford, *et al.* 1995; McKenzie, *et al.* 1997).

Although the biological functions of trefoil peptides are not completely understood, there is growing evidence supporting the involvement of these peptides in mucosal defence and reconstitution (Wright, *et al.* 1993; Dignass, *et al.* 1994; Babyatsky, *et al.* 1996; Mashimo, *et al.* 1996; Playford, *et al.* 1996). Data on record demonstrates that the expression of trefoil peptides (mainly TFF1 and TFF2) is up-regulated in epithelial cells adjacent to ulcerative conditions of the gastrointestinal tract and in epithelial cells

undergoing migration across the base of such lesions (UACL – ulcer associated cell lineage) (Wright, *et al.* 1990; Rio, *et al.* 1991; Wright, *et al.* 1993). Experimental data have shown that trefoil peptides are able to promote epithelial cell migration *in vitro* and *in vivo* (Dignass, *et al.* 1994; Playford, *et al.* 1995) and to protect against induced gastrointestinal damage *in vivo* (Babyatsky, *et al.* 1996; Playford, *et al.* 1996). On the other hand, a transgenic mouse model lacking TFF3 (ITF) has been shown to have impaired mucosal healing (Mashimo, *et al.* 1996).

There are two main theories regarding the mechanisms responsible for this protective function: one relies on the possibility that trefoil peptides act in a passive way, perhaps by enhancing the properties of the protective mucus gel covering the gastrointestinal epithelium; the other implies that trefoil peptides may be active via basolateral receptors, despite being secreted normally into the gut lumen, indicating that a receptor mediated response may be involved rather than a lumen-protective function.

Following the first hypothesis, it has been proposed that trefoil peptides and mucins may act in a synergistic manner to protect and reconstitute epithelial tissues (Dignass, *et al.* 1994; Otto and Wright, 1994; Kindon, *et al.* 1995). According to such a model, trefoil peptides and mucins would bind together to form a structure that could stabilise the viscoelastic gel of the protective mucus overlying the epithelium of the gut (Otto and Wright, 1994). This hypothesis is supported by data on the tri-dimensional structure of these molecules (Gajhede, *et al.* 1993; Carr, *et al.* 1994; De, *et al.* 1994) and on the co-expression of trefoil peptides and mucins (Poulsom and Wright, 1993; Wright, *et al.* 1993; Sands and Podolsky, 1996). TFF1 and TFF2 have been described as being co-expressed with MUC1 in human gastric mucosa (Wright, *et al.* 1993), and colon carcinoma cell lines that express MUC2 also secrete TFF2 and TFF3 (Giraud, *et al.* 1994). Curiously, in *X. laevis* several trefoil peptides are encoded by genes that also bear mucin core sequences (Hoffmann, 1988; Hauser, *et al.* 1990; Hauser and Hoffmann, 1992), highlighting the possibility of some sort of common ancestrality, although in mammals no homologues are known.

In accordance with the second hypothesis it has been shown that, in gastrointestinal cells, TFF3 can modulate epidermal growth factor (EGF) effects on epithelial ion

transport only when the basolateral surface of the cell is exposed to the peptide (Chinery and Cox, 1995). Furthermore, in *in vitro* cell models, TFF3 has been shown to be able to modulate its physiological function by at least two different mechanisms: induction of tyrosine phosphorylation of β-catenin and epidermal growth factor receptor (EGFr) (Liu, *et al.* 1997); and inhibition of tyrosine phosphorylation of ERK (extracellular signal-related protein kinase) (Kanai, *et al.* 1998). This capacity of signalling cytoplasmic proteins involved in signal transduction is highly suggestive of a receptor-mediated response, though no specific trefoil peptide receptors have been identified so far.

Recent results suggest that the ability of trefoil peptides to act as motogens might be mediated through modulation of the E-cadherin/catenin complex function (Hanby, *et al.* 1996; Liu, *et al.* 1997; Efstathiou, *et al.* 1998; Efstathiou, *et al.* 1999). E-cadherin has been found to be down-regulated in the reparative epithelium adjacent to ulcerative conditions of the gastrointestinal tract (Hanby, *et al.* 1996). Moreover, as mentioned before, in *in vitro* cell models TFF3 has been shown to modulate epithelial cell adhesion and migration through β-catenin tyrosine phosphorylation and consequent perturbation of the complexes between β-catenin, E-cadherin and associated proteins (Liu, *et al.* 1997; Efstathiou, *et al.* 1998). In a very recent report, Efstathiou *et al* (1999) demonstrated that transfection of full-length E-cadherin cDNA into an E-cadherin defective colon carcinoma cell line restores responsiveness to the migratory effects induced by TFF2.

Expression of trefoil peptides in cancer

The expression of trefoil peptides has been described in carcinomas of a variety of organs. Table 5 summarises data on record regarding the expression of human trefoil peptides in carcinomas of different organs, as well as in different types of putative premalignant lesions.

Table 5 – Expression of human trefoil peptides in carcinomas of different organs, as well as in different types of putative premalignant lesions.

Trefoil peptide	Organ	Description	References
TFF1	Stomach	Carcinoma: diffuse > intestinal	(Luqmani, *et al.* 1989; Henry, *et al.* 1991; Theisinger, *et al.* 1991; Müller and Borchard, 1993)
	Small intestine	Gastric metaplasia	(Hanby, *et al.* 1993a; Khulusi, *et al.* 1995)
	Large intestine	Carcinoma Hyperplastic polyps Adenoma	(Hanby, *et al.* 1993c; Welter, *et al.* 1994; Taupin, *et al.* 1996; Labouvie, *et al.* 1997)
	Pancreas	Carcinoma	(Welter, *et al.* 1992)
	Lung	Adenocarcinoma	(Higashiyama, *et al.* 1994)
	Endometrium	Carcinoma	(Henry, *et al.* 1991)
	Ovary	Mucinous carcinoma	(Dante, *et al.* 1994)
	Prostate	Carcinoma	(Bonkhoff, *et al.* 1995)
	Urinary bladder	Carcinoma	(Lipponen and Eskelinen, 1994)
	Gall bladder	Carcinoma	(Seitz, *et al.* 1991)
	Oesophagus	Carcinoma Barrett's metaplasia	(Hanby, *et al.* 1994; Labouvie, *et al.* 1999)
	Skin	Mucinous carcinoma	(Hanby, *et al.* 1998)
	Breast	Carcinoma	(Foekens, *et al.* 1990; Cappelletti, *et al.* 1992)
TFF2	Stomach	Carcinoma	(Theisinger, *et al.* 1991)
	Small intestine	Gastric metaplasia	(Hanby, *et al.* 1993a; Khulusi, *et al.* 1995)
	Large intestine	Adenoma Hyperplastic polyps	(Hanby, *et al.* 1993c; Taupin, *et al.* 1996)
	Pancreas	Carcinoma	(Welter, *et al.* 1992)
	Gall bladder	Carcinoma	(Theisinger, *et al.* 1991)
	Oesophagus	Barrett's metaplasia	(Hanby, *et al.* 1994; Labouvie, *et al.* 1999)
TFF3	Large intestine	Carcinoma Adenoma Hyperplastic polyps	(Hanby, *et al.* 1993c; Taupin, *et al.* 1996)
	Skin	Mucinous carcinoma	(Hanby, *et al.* 1998)
	Breast	Carcinoma	(Theisinger, *et al.* 1996; May and Westley, 1997; Poulsom, *et al.* 1997)

The most widely studied trefoil peptide in cancer is TFF1, probable because it was the first human trefoil peptide to be discovered and because it was discovered in tumour cells of the breast carcinoma cell line MCF-7 (Masiakowski, *et al.* 1982). In human breast carcinoma, TFF1 expression can be detected in more than 50% of the tumours and is associated, though not exclusively, with oestrogen receptor expression (Rio, *et al.* 1987; Skilton, *et al.* 1989; Pallud, *et al.* 1993), responsiveness to hormone therapy (Henry, *et al.* 1990) and favourable prognosis (Foekens, *et al.* 1990; Cappelletti, *et al.* 1992). Other tumours found to express TFF1 include carcinomas occurring in different

organs: pancreas (Welter, *et al.* 1992), lung (Higashiyama, *et al.* 1994), endometrium (Henry, *et al.* 1991), ovary (mucinous carcinoma) (Dante, *et al.* 1994), prostate (Bonkhoff, *et al.* 1995), urinary bladder (Lipponen and Eskelinen, 1994), billiary tract (Seitz, *et al.* 1991), colorectum (Welter, *et al.* 1994; Labouvie, *et al.* 1997), oesophagus (Labouvie, *et al.* 1999) and skin (mucinous carcinoma) (Hanby, *et al.* 1998) (Table 5). In gastric carcinoma the expression of TFF1 has been described by several authors (Luqmani, *et al.* 1989; Henry, *et al.* 1991; Theisinger, *et al.* 1991; Müller and Borchard, 1993) in a frequency ranging from 48% to 57%. Theisinger *et al* (1991) reported an association between the expression of TFF1 and the diffuse type of gastric carcinoma and Müller and Borchard (1993) found an association between TFF1 expression and the extent of tumour growth.

TFF2 expression in human carcinomas has been less extensively studied, but expression of this trefoil peptide has been reported in stomach (Theisinger, *et al.* 1991), billiary tract (Seitz, *et al.* 1991) and pancreatic carcinoma (Welter, *et al.* 1992) (Table 5).

TFF3 is the most recently discovered trefoil peptide, and this fact is reflected in the scarce number of studies on its expression in human malignancies: TFF3 expression has been detected in colorectal carcinomas, associated with loss of differentiation and co-localising with mucin (Taupin, *et al.* 1996); in skin mucinous carcinoma (Hanby, *et al.* 1998); as well as in breast carcinoma (Theisinger, *et al.* 1996; May and Westley, 1997; Poulsom, *et al.* 1997), where it co-localises with TFF1 (Poulsom, *et al.* 1997) (Table 5). TFF3 expression in breast, like expression of TFF1, is under control of oestrogen receptor (May and Westley, 1997).

Besides their expression in human carcinomas and in ulcerative conditions of the gastrointestinal tract, trefoil peptides were shown to be expressed in several types of benign neoplastic lesions of the gastrointestinal epithelium. In Barrett's oesophagus (Hanby, *et al.* 1994; Labouvie, *et al.* 1999) and in duodenal gastric metaplasia (Hanby, *et al.* 1993a; Khulusi, *et al.* 1995) both TFF1 and TFF2 expression was observed with a pattern resembling that of the native gastric epithelium and UACL: expression of TFF1 in the upper ducts and surface cells and expression of TFF2 in acinar cells and lower ducts.

In colorectal hyperplastic polyps and adenomas, the expression of all three trefoil peptides has been detected (Hanby, *et al.* 1993c; Taupin, *et al.* 1996) (Table 5); interestingly, and similarly to what has been described for TFF2 in gastric foveolar epithelium (Hanby, *et al.* 1993b), TFF2 and TFF3 mRNA is detectable in colorectal hyperplastic polyps despite the absence of expression of the respective proteins.

Despite all the evidence pointing to an association between altered trefoil peptides expression and cancer development, the biological role of these proteins in tumourigenesis remains unclear. Experimental evidence for a role in tumour suppression comes from studies using knockout mice lacking the *BCEI* gene (Lefebvre, *et al.* 1996). Homozygous animals showed decreased and dysfunctional gastric mucin production with marked antral hyperplasia and dysplasia; all such animals developed antral adenomas and 30% developed multifocal intramucosal carcinomas. Recently, Familari *et al* (1998) demonstrated that trefoil peptides are early markers of epithelial cell maturation in the developing rat gut and suggested they may play a role in epithelial cell differentiation. These findings suggest that the *BCEI* gene rather than acting directly in tumour suppression at a genetic level may act, indirectly, *via* maintenance of a normal tissue differentiation.

It remains to be seen if the ability of trefoil peptides to act as motogens may play any role in the mechanisms of invasion and metastisation of neoplastic cells. Noteworthy, among several cancer types surveyed, carcinoma of the pancreas — a very aggressive type of tumour — has the highest rate of TFF1-positive tumours (Henry, *et al.* 1991; Welter, *et al.* 1992). In breast cancer, however, in which TFF1 has been studied in great detail, no convincing evidence was provided regarding a putative relationship between TFF1 expression and invasive/metastatic abilities of the tumours (Poulsom, *et al.* 1997).

It seems clear that tumours presenting alteration of trefoil peptides expression should be divided in two main groups: those arising in organs without native expression of trefoil peptides, where the expression of these proteins constitutes a *de novo* event; and those arising in organs in which trefoil peptides are constitutivelly expressed, where the tumours either maintain or exhibit a (partial or complete) loss of the expression of these

proteins. Although the biological importance of trefoil peptides expression remains unclear for both groups, probably it is different in each of them.

E-cadherin

General features

E-cadherin belongs to the cadherin family of calcium dependent cell adhesion molecules (Takeichi, 1991). Together with other families of cell adhesion molecules, the cadherins play an essential role in biological processes such as ordering of cell sorting, migration and differentiation, regulation of inter- and intracellular signalling and control of gene transcription (Takeichi, 1991; Kemler, 1993; Ilyas and Tomlinson, 1997).

Classically, three main groups of cadherins have been defined: E-cadherin, found in epithelial tissues and also known as uvomorulin, cell CAM 120/80, Arc-1 or L-CAM (Nagafuchi, *et al.* 1987; Mansouri, *et al.* 1988); P-cadherin, found primarily in placenta (Nose, *et al.* 1987; Shimoyama, *et al.* 1989); and N-cadherin, found in neural tissues and muscle (Hatta, *et al.* 1988; Miyatani, *et al.* 1989). This initial classification has rapidly expanded along with the identification of other molecules (e.g. M-cadherin, desmoglein and desmocollin), which share protein sequence similarities with cadherins (Heimark, *et al.* 1990; Koch, *et al.* 1990; Donalies, *et al.* 1991; Buxton and Magee, 1992). Each of the subclasses of cadherins displays a unique pattern of tissue distribution and, in many types of cells, several types of cadherins are co-expressed in varying combinations (Takeichi, 1988; Takeichi, 1990).

Cadherins are transmembrane cell-cell adhesion molecules that bind to one another by means of homophilic interactions (Takeichi, 1991). Intracellularly, they attach to catenins which link the cytoplasmic domain of the cadherins to intermediate filaments of the cytoskeleton (Fig. 4) (Kemler, 1993). The expression of these molecules is developmentally regulated and the segregation and remodelling of embryonic tissues is associated with sequential expression of different cadherin molecules (Takeichi, 1988; Takeichi, 1990). The importance of cadherins in establishing and maintaining intercellular connections is reinforced by the fact that as long as cadherins are

functioning, inactivation of other adhesion systems seems to have little effect on cell-cell adhesion (Duband, *et al.* 1987; Larjava, *et al.* 1990).

Cadherins are involved both in junctional and nonjunctional adhesion mechanisms and are localised in lateral cell-cell contacts and enriched in the *zonula adherens* and desmosomal junctions (Fig. 4).

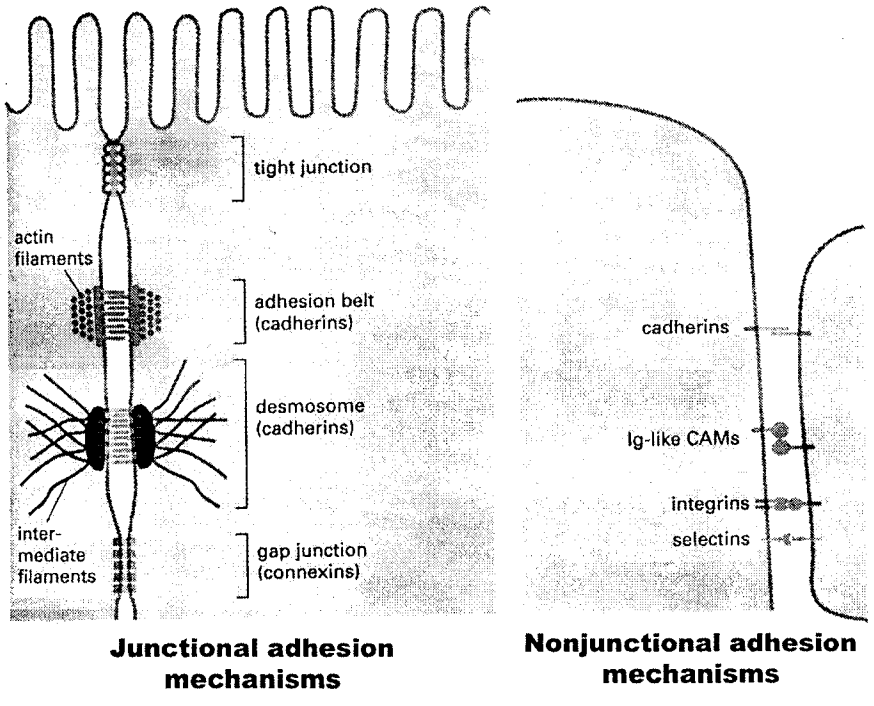


Fig. 4 – In junctional cell-cell adhesion mechanisms, cadherins are localised in *zonula adherens*, forming the adhesion belt, and in desmosomes; in nonjunctional adhesion mechanisms cadherins are dispersed along the lateral cell membrane with other types of cell adhesion molecules. The extracellular part of the cadherin molecules binds homophilically to one another and the cytoplasmic part is connected to intermediate filaments through catenins. Adapted after Alberts *et al* (1994).

Expression of E-cadherin in normal tissues

The cell-cell adhesion molecule E-cadherin plays an essential role in morphogenesis and in the formation and maintenance of the normal architecture and function of epithelial tissues (Takeichi, 1991; Takeichi, 1995). Studies conducted in mice models show that,

in early development, E-cadherin first functions as an adhesion component during compaction of blastomers (Hyafil, *et al.* 1981; Takeichi, 1991); at later stages, it is confined to epithelial tissues, localised in lateral cell-cell contacts and enriched in the *zonula adherens* (Eidelman, *et al.* 1989; Takeichi, 1991).

Structure of the E-cadherin gene and protein

The human E-cadherin locus, denominated *CDH1*, contains 16 exons (Fig. 5), bridges a region of 100 kbp (Berx, *et al.* 1995b) and maps on chromosome 16q22.1 (Mansouri, *et al.* 1988; Natt, *et al.* 1989; Berx, *et al.* 1995b).

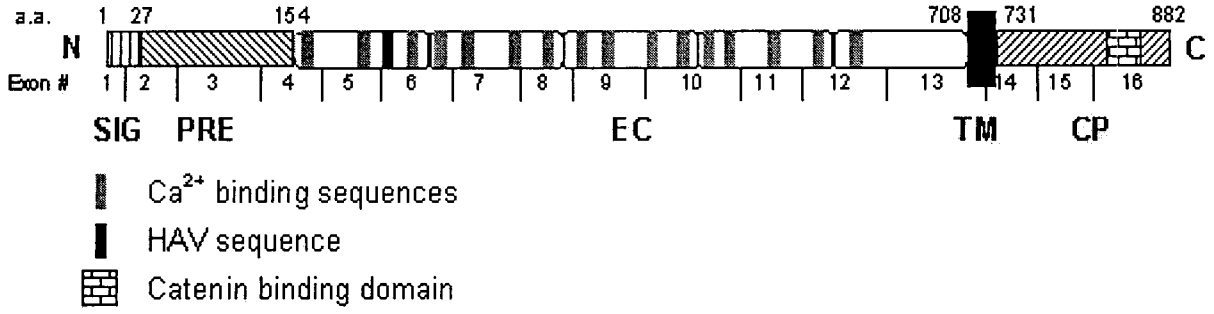


Fig. 5 – Schematic structure of the E-cadherin protein. In its precursor form E-cadherin is 882 amino acids long; after cleavage of the signalling sequence (SIG) and precursor sequence (PRE), the mature form of E-cadherin encompasses 729 amino acids and contains a cytoplasmic part (CP), a transmembrane domain (TM), and an extracellular part (EC), the latter with five repeated cadherin domains. The exonic structure of the E-cadherin mRNA is marked below the drawing. Adapted after Berx *et al* (1995a).

E-cadherin is synthesised from a 4.5 kb mRNA as a 135 kDa precursor polypeptide which is rapidly (two hours) and efficiently (100%) processed to the mature 120 kDa form (Shore and Nelson, 1991). It is delivered in its mature form to the cell surface, where it exists as a dimer and has a half-life of about 5 hours (Shore and Nelson, 1991). The mature E-cadherin is an integral membrane glycoprotein with a single membrane spanning domain and an extracellular part (N-terminal) that is implicated in homophilic binding (Fig. 5); the cytoplasmic domain (C-terminal) is noncovalently linked to catenins through a catenin binding domain (Fig. 5) (Kemler, 1993).

The extracellular part of E-cadherin is largely composed of five homologous repeated domains, known as cadherin domains, each containing about 110 amino acid residues (Fig. 5) (Kemler, 1993). Each cadherin domain contains two highly conserved calcium binding motifs, formed by four calcium binding sequences (Fig. 6) and found at similar locations in all cadherins (Kemler, 1993). These motifs play a key role in the correct folding and dimerisation of these proteins as well as in the adhesion mechanism itself (Overduin, *et al.* 1995; Shapiro, *et al.* 1995; Nagar, *et al.* 1996). The homophilic binding specificity of cadherins appears to be governed by their N-terminal cadherin domain through a surface that includes the HAV motif (Overduin, *et al.* 1995) (Figs. 5 and 6); however, recent data demonstrates that besides homophilic homotypic interactions, E-cadherin can also establish homophilic heterotypic (Tang, *et al.* 1993) and heterophilic heterotypic (Cepek, *et al.* 1994) interactions.

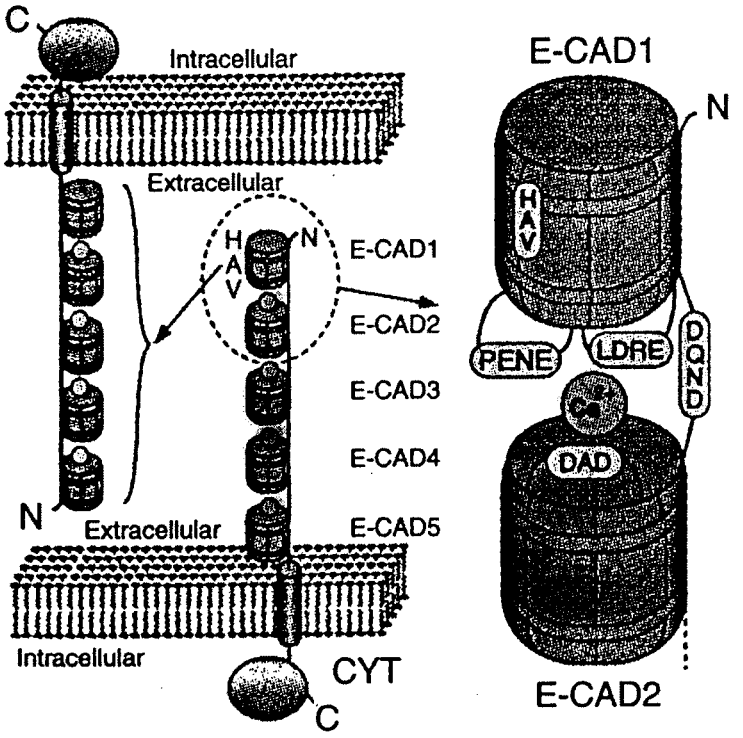


Fig. 6 – A schematic drawing of E-cadherin molecules presented by two cells. On the left side cadherin domains are depicted as barrels and calcium ions are represented by spheres that are sandwiched between tandem cadherin domains by the four calcium binding sequences (DAD, DQND, PENE and LDRE) seen in the enlargement of cadherin domains 1 and 2 on the right side. The HAV-containing surface on the

cadherin 1 domain contributes to homophilic binding specificity between cadherins. C, cytoplasmic domain; CYT, cytoplasm; E-CADn, cadherin domains. Adapted after Overduin *et al* (1995).

The role of E-cadherin in the epithelial cell-cell adhesion system

E-cadherin is only part of a complex cell adhesion system and its function is dependent on the interaction of its conserved cytoplasmic domain with the cytoskeleton *via* molecular complexes involving α-, β- and γ-catenin (Fig. 7). Both β-catenin and γ-catenin/plakoglobin are members of the armadillo protein family (Aberle, *et al.* 1996) and link E-cadherin via α-catenin (member of the vinculin family) to the actin cytoskeleton. Another catenin, $p120^{ctn}$, which was originally identified as a substrate of Src and several receptor tyrosine kinases, also interacts with the cytoplasmic domain of E-cadherin, but its function remains unclear (Fig. 7) (Reynolds, *et al.* 1994). This mechanism allows E-cadherin to be directly involved in the cell-cell adhesion process and in nuclear signalling *via* catenins and the cytoskeleton; furthermore, E-cadherin participates in the regulation of the levels of β-catenin in the cytoplasm, which in turn functions in the Wnt signal transduction pathway (Miller and Moon, 1996; Barth, *et al.* 1997; Gumbiner, 1997).

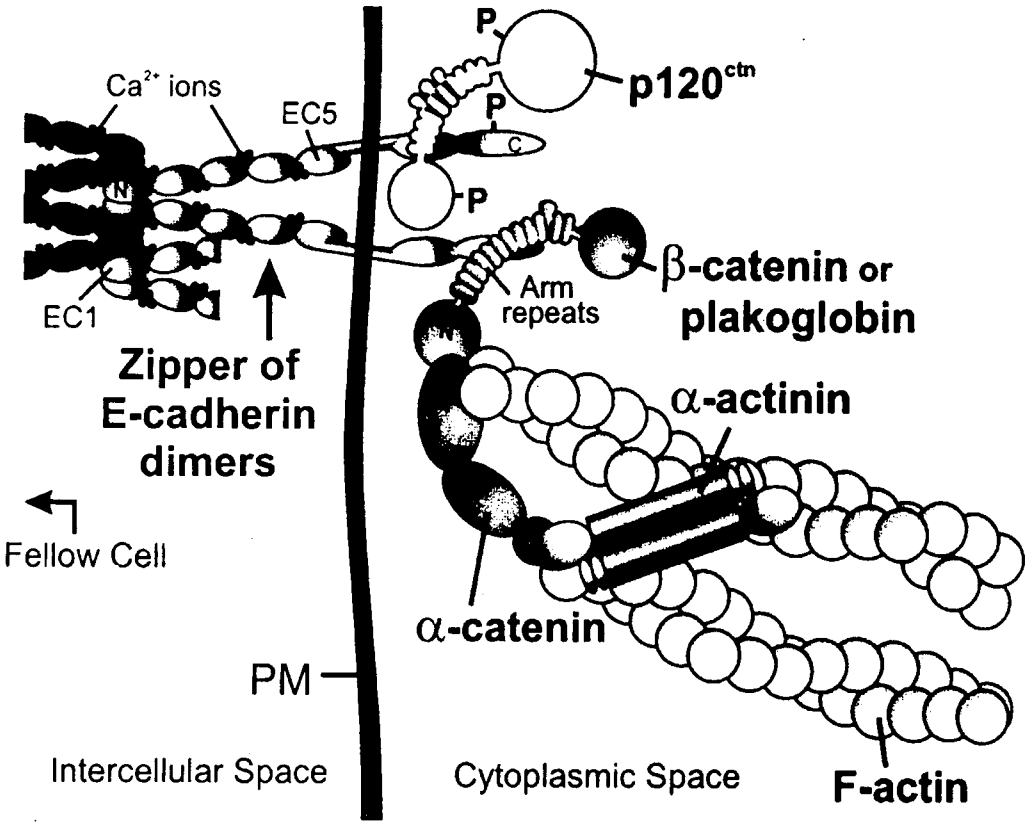


Fig. 7 – Schematic overview of the E-cadherin-catenin-cytoskeleton complex at the plasma membrane (PM) of two neighbouring cells. The N-terminal ends of E-cadherin dimers extend into the intercellular space, where they interact in a homophilic zipper-like fashion with similar E-cadherin dimers (dark globules), extending from the opposing cell surface. Calcium ions are essential for the correct conformation and functionality of the cadherins. The C-terminal ends of the E-cadherin molecules extend into the cytoplasm. These domains associate with the catenins (β-catenin, γ-catenin/plakoglobin, $p120^{ctn}$). Disk-like structures in the latter molecules represent armadillo tandem repeats. The β-catenin and plakoglobin molecules in turn associate with α-catenin. This latter forms the molecular link to the F-actin microfilaments, either directly or *via* α-actinin. P, phosphorylation on Ser or Tyr residues. Adapted after Vermeulen *et al* (1996) and Berx *et al* (1998).

Loss of E-cadherin function in human cancer

Given the essential role of E-cadherin in the processes of cellular adhesion, migration and growth control, it has been postulated that changes in expression or function of E-cadherin may account for the ability of cancer cells to detach from the parent tumour structure and invade locally. In fact, aberrant expression, reduction and/or loss of expression of E-cadherin have been observed in several types of carcinoma, and frequently associated with poorly differentiated/undifferentiated carcinomas and/or

invasive tumours, including carcinomas of the colon (Dorudi, *et al.* 1993), prostate (Umbas, *et al.* 1997), breast (Moll, *et al.* 1993), bladder (Bringuier, *et al.* 1993; Hong, *et al.* 1995), pancreas (Pignatelli, *et al.* 1994), oesophagus (Jankowski, *et al.* 1994; Krishnadath, *et al.* 1997), stomach (Mayer, *et al.* 1993; Shino, *et al.* 1995; Gabbert, *et al.* 1996; Jawhari, *et al.* 1997), head and neck (Schipper, *et al.* 1991), cervix (Vessey, *et al.* 1995) and thyroid (Soares, *et al.* 1997).

Loss of expression of E-cadherin has been associated with mutations of the E-cadherin gene *CDH1* and/or loss of heterozygosity (LOH) in 16q22.1, that have been reported in diffuse gastric carcinoma (Becker, *et al.* 1994; Muta, *et al.* 1996; Guilford, *et al.* 1998), lobular carcinoma of the breast (Berx, *et al.* 1995a; Berx, *et al.* 1996), colon carcinoma (Efstathiou, *et al.* 1999) and carcinomas of the endometrium and ovary (Risinger, *et al.* 1994). These findings support a role of E-cadherin as a tumour and/or invasion suppressor gene, as previously suggested by *in vitro* observations demonstrating that transfection of invasive E-cadherin-negative cell lines with vectors which express fully functional E-cadherin results in loss of invasive features (Frixen, *et al.* 1991; Vleminckx, *et al.* 1991).

Loss of E-cadherin function can, however, occur in the absence of *CDH1* mutation: mutation of other members of the cadherin/catenin complex, such as α- and β-catenin, can abolish E-cadherin mediated adhesion (Morton, *et al.* 1993; Oyama, *et al.* 1994); and phosphorylation of β-catenin renders it useless for cell adhesion. This later effect has been shown for EGFr (Hoschuetzky, *et al.* 1994), c-*erb*B2 (Ochiai, *et al.* 1994), hepatocyte growth factor receptor *MET* (Shibamoto, *et al.* 1994), the oncoprotein $p60^{src}$ (Matsuyoshi, *et al.* 1992; Behrens, *et al.* 1993), the mucin MUC1 (Yamamoto, *et al.* 1997; Li, *et al.* 1998) and the trefoil peptide TFF3 (Liu, *et al.* 1997; Efstathiou, *et al.* 1998) which can either phosphorylate or induce phosphorylation of β-catenin; in addition, transcriptional silencing of *CDH1* by methylation of the promoter leads to down-regulation of E-cadherin expression in some tumours (Graff, *et al.* 1995; Hennig, *et al.* 1995; Yoshiura, *et al.* 1995).

In support of an important role of the E-cadherin/catenin complex during tumour development is the interaction of β-catenin with the adenomatous polyposis coli (APC)

tumour suppressor protein (Su, *et al.* 1993). Truncation mutants of APC, as frequently found in colon tumours, loose their ability to regulate β-catenin and to trigger its degradation, resulting in elevated cytoplasmic and nuclear concentrations of β-catenin. This, in turn, leads to modification of gene expression patterns, e.g. *C-MYC* (He, *et al.* 1998), by a complex of β-catenin with members of the TCF/LEF1 transcription factor family (Behrens, *et al.* 1996).

Altogether, the data on record regarding the involvement of E-cadherin in different types of tumours support the existence of a close association between loss of function of E-cadherin and infiltrative/invasive growth pattern of the tumours with reduced or no adhesion between cancer cells, features that are shared by diffuse gastric carcinoma (Mayer, *et al.* 1993; Yonemura, *et al.* 1995).

The introduction of the Thesis highlights that one of the most striking features of gastric carcinoma is its morphologic heterogeneity and consequent diversity of biological behaviours. Among the many structural patterns recognised in gastric carcinoma (Borrman, 1926; Laurén, 1965; Ming, 1977; Watanabe, *et al.* 1991; Carneiro, *et al.* 1995b), two morphologic entities — with distinct epidemiological, histogenetic and clinicopathological profiles — emerge: the so-called intestinal and diffuse carcinoma. At variance with this, a relatively broad group of gastric carcinomas, displaying either a mixed structure — encompassing different structural components — or exhibiting a solid architecture, remain poorly characterised.

Regarding cell differentiation, data from ultrastructural studies (Fiocca, *et al.* 1987; Carneiro, *et al.* 1992; Carneiro, 1997) and from histochemical and immunohistochemical analyses (Bara, *et al.* 1981; Tatematsu, *et al.* 1986; Fiocca, *et al.* 1988a; Fiocca, *et al.* 1988b; Fiocca, *et al.* 1990; Kushima, *et al.* 1993; Kushima and Hattori, 1993; Carneiro, 1997; Yamachika, *et al.* 1997) show that gastric carcinoma exhibits, alone or in combination, two main types of cell differentiation — gastric and intestinal.

When we started this study, in 1994, several molecular changes had been identified in gastric carcinoma (Lemoine, *et al.* 1992; Seruca, *et al.* 1992; Wright and Williams, 1993; Carneiro, *et al.* 1994; Correa and Shiao, 1994; David, *et al.* 1994; Seruca, *et al.* 1995; Santos, *et al.* 1996; Tahara, 1995; Amado, *et al.* 1998), suggesting that there is a molecular counterpart for each of the two main types of gastric carcinoma. However, by that time, the molecular change(s) underneath the establishment of the two distinct structural patterns of gastric carcinoma — gland-forming or diffuse growth pattern — were not clearly identified.

Taking all these aspects into account, the present study was elaborated in order to address the following four topics:

1 – Study of the pattern of cell differentiation along the process of gastric carcinogenesis.

In this part of the study we aimed at characterising the cell differentiation patterns of gastric carcinoma and different premalignant lesions of the stomach, namely chronic atrophic gastritis, intestinal metaplasia and gastric polyps, in an attempt to see if this approach might contribute to the clarification of the histogenesis and cell differentiation of gastric carcinoma.

In order to accomplish this goal we evaluated the expression patterns of the two stomach specific trefoil peptides (TFF1 and TFF2) in the aforementioned lesions. Furthermore, the expression patterns of gastric trefoil peptides were co-analysed with those of two gastric mucins MUC5AC and MUC6, the intestinal mucin MUC2 and the more ubiquitous MUC1 mucin.

2 – Analysis of molecular events underlying the intestinal and diffuse histotype of gastric carcinoma.

Shortly before the starting of the present study, alterations at the structural and expression levels of E-cadherin were described in sporadic diffuse-type gastric carcinoma (Becker, *et al.* 1993; Becker, *et al.* 1994), suggesting that this molecule could play a major role in the carcinogenesis of diffuse carcinoma.

Having this in mind we analysed E-cadherin changes in different types of gastric carcinoma. Our aim was twofold: to study the patterns of expression of E-cadherin at the protein level and to clarify, at the gene level, the alterations behind altered expression; to see, whether or not, the study of E-cadherin changes could shed some light into the elucidation of the molecular mechanisms underlying the dichotomic structural pattern of gastric carcinoma, expressed in its two main histotypes — intestinal and diffuse.

3 – Analysis of some molecular and phenotypic features underlying mixed gastric carcinoma.

The morphologic heterogeneity of gastric carcinomas is also reflected in individual cases; many gastric carcinomas are pluriform, displaying two or more distinct components. Most classifications overcome this aspect by grouping gastric carcinomas according to the predominant component of the tumours (Nakamura, *et al.* 1968; Mulligan, 1972; Watanabe, *et al.* 1991) and only a few authors stress the existence of gastric carcinomas with mixed morphologic patterns (Laurén, 1965; Carneiro, *et al.* 1995b).

As a consequence, the available knowledge on the biopathology of mixed gastric carcinoma is scarce and it is still unknown if these tumours derive from carcinomas with a "pure" histological type (and, if yes, from which type) or if they originate *ab initio* as mixed carcinomas.

In an attempt to contribute to the clarification of the nature of mixed gastric carcinomas, we performed a comparative study of the intestinal and diffuse histological components of these tumours. The comparison was focused on the analysis of cell differentiation (expression pattern of trefoil peptides and mucins) and on the search of molecular events putatively related with the phenotypic (structural) divergence of the tumours (E-cadherin alterations).

4 – Re-evaluation of the histogenetic pathways leading to intestinal, diffuse and mixed gastric carcinoma.

Finally, our ultimate goal was to integrate all the information drawn from the aforedescribed studies and to provide additional evidence in order to progress in the understanding of the histogenetic pathways leading to intestinal, diffuse and mixed gastric carcinoma.

MATERIAL AND METHODS

The methods used in the different studies are described in detail in Papers I to VI. Briefly, the following methods were used:

Immunohistochemistry (IHC) – Avidin-biotin-peroxidase method used in the studies of the expression of the trefoil peptides TFF1 (Papers I – IV) and TFF2 (Papers III and IV), the mucins MUC1, MUC2, MUC5AC and MUC6 (Papers III and IV) and E-cadherin (Paper V).

Histochemical methods - Periodic acid Schiff (PAS), alcian blue pH 2.5/PAS (AB/PAS) and alcian blue pH 2.5/high iron diamine (HID/AB), used in the histochemical characterisation of mucins (Paper I).

Immunoradiometric assay (IRMA) – Used for the quantification of TFF1 protein expression (Paper II).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) – Used for the semi-quantification of TFF1 and TFF2 mRNA expression (Paper III).

Polymerase Chain Reaction (PCR) – Used in the LOH analysis and mutation screening of the *CDH1*/E-cadherin gene (Paper VI).

Single Strain Conformation Polymorphism (SSCP) – Used in the mutation screening of the *CDH1*/E-cadherin gene (Paper VI).

Sequencing – Used in the automated DNA sequencing of the *CDH1*/E-cadherin gene (Paper VI).

Statistical analysis – Pearson χ^2 test (papers I - VI), unpaired Student's *t*-test (Paper II), Mann-Whitney *U* test (Paper II), Kaplan-Meier survival curves (Papers II and V), Mantel-Cox test (Papers II and V), Cox's proportional hazards model (Paper V) and MPLR (maximum partial likelihood ratio) method (Paper V).

The biological material included in the different studies is described in papers I-VI; due to the diversity of the lesions included in each of these studies, their description will now be omitted.

RESULTS

The results are documented in detail in papers I to VI. In this section, a summary will be presented stressing the most interesting findings in relation to the issues raised in the Aims section.

Expression of TFF1 in premalignant lesions of the stomach and in gastric carcinoma (Papers I and II).

Normal mucosa and chronic atrophic gastritis

TFF1 was expressed throughout foveolar and superficial epithelium of normal gastric mucosa and this pattern was retained in chronic atrophic gastritis, out of intestinal metaplasia (IM) lesions.

Intestinal metaplasia

In IM, TFF1 expression was observed in every case. Furthermore, there was a clear difference between complete and incomplete IM types: in complete type IM, TFF1 expression was restricted to goblet cells, whereas in incomplete type IM TFF1 was expressed both in goblet and columnar cells.

Gastric polyps

In hyperplastic polyps of the stomach, TFF1 was expressed in every case (Table 6). In gastric adenomatous polyps, TFF1 expression was found in eight out of 11 cases (72.7%) (Table 6).

The percentage of cases expressing TFF1 in the majority of cells was significantly higher ($p=0.0002$) in hyperplastic polyps than in adenomatous polyps (Table 6).

Table 6: Relationship between TFF1 immunoreactivity and gastric polyp type.

Polyps	% of immunoreactive cells		
	-	<50%	>50%
Hyperplastic (n=10)	0 (0)	0 (0)	10(100)
Adenomatous (n=11)	3 (27.3)	7 (63.6)	1 (9.1)

p value=0.0002

Gastric carcinoma

In gastric carcinomas, TFF1 immunostaining was observed in 33 out of 50 cases (66.0%): in 16 out of 18 (88.9%) diffuse carcinomas and in 15 out of 28 (53.6%) intestinal carcinomas. This difference was found to be statistically significant (p=0.037) (Table 7).

Table 7: Relationship between the histological type of gastric carcinomas and TFF1 expression.

Histological type	No. of cases (%)	TFF1 IHC		p value
		Negative	Positive	
Intestinal	28 (56.0)	13 (46.4)	15 (53.6)	
Diffuse	18 (36.0)	2 (11.1)	16 (88.9)	0.037
Unclassifiable	4 (8.0)	2 (50.0)	2 (50.0)	
Total	50 (100)	17 (34)	33 (66)	

Co-expression of trefoil peptides (TFF1 and TFF2) and mucins (MUC1, MUC2, MUC5AC and MUC6) in gastric polyps and in gastric carcinoma (Papers III and IV).

Gastric polyps

In hyperplastic polyps, TFF1, MUC1 and MUC5AC were detected in every case. TFF2 expression was not observed in any case. Two cases presented focal MUC2 expression and MUC6 immunoreactivity was detected in another two cases (Table 8).

In adenomatous polyps, TFF1 expression was observed in 16 cases (84.2%); TFF2 expression was not detected in any case; MUC1 was detected in 14 cases (73.7%); MUC2 expression was found in seven cases (36.8%); MUC5AC expression was observed in nine cases (47.4%), all of them co-expressing TFF1 and MUC1; and MUC6 was expressed in 7 cases (36.8%), all of them displaying also TFF1, MUC1 and MUC5AC immunoreactivity (Table 8).

Table 8- Pattern of expression of trefoil peptides and mucins in hyperplastic and adenomatous polyps.

	Number of positive cases (%)					
Polyps	**TFF1**	**TFF2**	**MUC1**	**MUC2**	**MUC5AC**	**MUC6**
Hyperplastic (n=10)	10 (100%)	0 (0%)	10 (100%)	2 (20%)	10 (100%)	2 (20%)
Adenomatous (n=19)	16 (84.2%)	0 (0%)	14 (73.7%)	7 (36.8%)	9 (47.4%)	7 (36.8%)

Gastric carcinoma

TFF1 and TFF2 expression was observed, respectively, in 64 cases (66.7%) and in 10 cases (10.4%) of 96 gastric carcinomas; MUC1 was detected in 79 cases (82.3%);

MUC2 immunoreactivity was seen in 22 cases (22.9%); expression for MUC5AC was detected in 61 cases (63.5%); and MUC6 immunoexpression was found in 36 cases (37.5%) (Table 9).

Table 9 - Comparison between the immunoexpression pattern of trefoil peptides and mucins and the histological type of 96 gastric carcinomas.

Histological type	IHC positive cases (%)					
	TFF1	**TFF2**	**MUC1**	**MUC2**	**MUC5AC**	**MUC6**
Diffuse ca (n = 33)	25 (75.8)	5 (15.2)	24 (72.7)	8 (24.2)	23 (69.7)	8 (24.2)
Intestinal ca (n = 53)	30 (56.6)	4 (7.5)	46 (86.8)	11 (20.8)	29 (54.7)	22 (41.5)
Unclassifiable ca (n = 10)	9 (90.0)	1 (10.0)	9 (90.0)	3 (30.0)	9 (90.0)	6 (60.0)
p value	0.05	NS	NS	NS	0.07	NS
Total (n = 96)	64 (66.7)	10 (10.4)	79 (82.3)	22 (22.9)	61 (63.5)	36 (37.5)

NS, Not significant.

The comparison between the immunohistochemical expression of the antigens under study and the histological classification of the tumours revealed a significant association (p=0.05) between the expression of TFF1 and the diffuse and unclassifiable histological types (Table 9). A suggestive association (p=0.07) was observed between MUC5AC expression and the diffuse and unclassifiable histotype (Table 9). No relationship was found between the expression of TFF2, MUC1, MUC2 or MUC6 and the histotype of the tumours.

Pattern of cell differentiation

The definition of the patterns of cell differentiation was based upon the expression of trefoil peptides and mucins typically expressed in the superficial zone of normal gastric mucosa. Three main phenotypes were defined: 1) complete gastric phenotype — co-expression of TFF1 and MUC5AC regardless of the expression of TFF2 and MUC6; 2) incomplete gastric phenotype — expression of either TFF1 or MUC5AC regardless of

the expression of TFF2 and MUC6; 3) non-gastric phenotype — no expression of TFF1, TFF2, MUC5AC and MUC6.

Gastric polyps

All hyperplastic polyps exhibited a complete gastric phenotype (Table 10). In adenomatous polyps three main phenotypes could be identified: complete gastric phenotype — 9 cases (47.4%); incomplete gastric phenotype — 7 cases (36.8%); and non-gastric phenotype — 3 cases (15.8%) (Table 10).

Table 10 - Pattern of cellular differentiation in hyperplastic and adenomatous polyps.

	Phenotype (%)		
Polyps	**Complete gastric**	**Incomplete gastric**	**Non-gastric**
Hyperplastic (n=10)	10 (100%)	0 (0%)	0 (0%)
Adenomatous (n=19)	9 (47.4%)	7 (36.8%)	3 (15.8%)

Gastric carcinoma

According to the expression pattern of trefoil peptides and mucins, 50 cases (52.1%) presenting co-expression of TFF1 and MUC5AC were identified as complete gastric-type. Incomplete gastric phenotype was observed in 28 cases (29.2%). Lack of expression of TFF1, TFF2, MUC5AC and MUC6 was found in the remaining 18 cases (18.7%), therefore identified as non-gastric phenotype (Table 11).

Table 11 – Comparison between phenotypic differentiation and histological type of gastric carcinomas.

Histology		Phenotype (%)			
	Cases (%)	Complete Gastric	Incomplete gastric	Non-gastric	p value
Diffuse ca	33 (34.4)	18 (54.5)	12 (36.4)	3 (9.1)	
Intestinal ca	53 (55.2)	24 (45.3)	14 (26.4)	15 (28.3)	0.06
Unclassifiable ca	10 (10.4)	8 (80.0)	2 (20.0)	0 (0)	
Total	96 (100)	50 (52.1)	28 (29.2)	18 (18.7)	

The prevalence of MUC1 expression was similar in the three phenotypes: in 44 out of 50 cases (88.0%) with complete gastric-type differentiation; in 22 out of 28 cases (78.6%) presenting incomplete gastric-type differentiation; and in 13 out of 18 cases (72.2%) classified as non-gastric phenotype. The same holds true regarding the expression of MUC2, which was seen in 14 out of 50 cases (28.0%) displaying complete gastric phenotype; in six out of 28 cases (21.4%) presenting incomplete gastric-type differentiation; and in two out of 18 cases (11.1%) with non-gastric phenotype.

The comparison between the histological type of the cases and the phenotype disclosed a suggestive association (p=0.06) between the diffuse and unclassifiable histotypes and the gastric (complete and incomplete) phenotype, and between the intestinal histotype and non-gastric type of differentiation (Table 11).

Most of the early tumours were identified as complete gastric-type (8/13 – 61.5%) or incomplete gastric-type (4/13 – 30.8%) and only one early tumour, with intestinal histotype, was found to present a non-gastric phenotype (7.7%) (Table 12); in contrast to this, in the group of advanced tumours, 17 out of 83 cases (20.5%) had a non-gastric phenotype (Table 12); these differences did not attain, however, the threshold of statistical significance (p=0.53) (Table 12). The same holds true regarding MUC2 expression, which was found to be more prevalent, though not significantly, in advanced tumours than in early tumours (p=0.16) (Table 13).

Table 12 – Comparison between phenotypic differentiation and extent of tumour growth of gastric carcinomas, according to the histological type.

T stage		Phenotype (%)			
	Cases (%)	Complete Gastric	Incomplete gastric	Non-gastric	p value
Diffuse					
Early	5 (15.2)	3 (60.0)	2 (40.0)	0	NS
Advanced	28 (84.8)	15 (53.5)	10 (35.7)	3 (10.7)	
Intestinal					
Early	8 (15.1)	5 (62.5)	2 (25.0)	1 (12.5)	NS
Advanced	45 (84.9)	19 (42.2)	12 (26.7)	14 (31.1)	
Total					
Early	13 (13.5)	8 (61.5)	4 (30.8)	1 (7.7)	NS
Advanced	83 (86.5)	42 (50.6)	24 (28.9)	17 (20.5)	

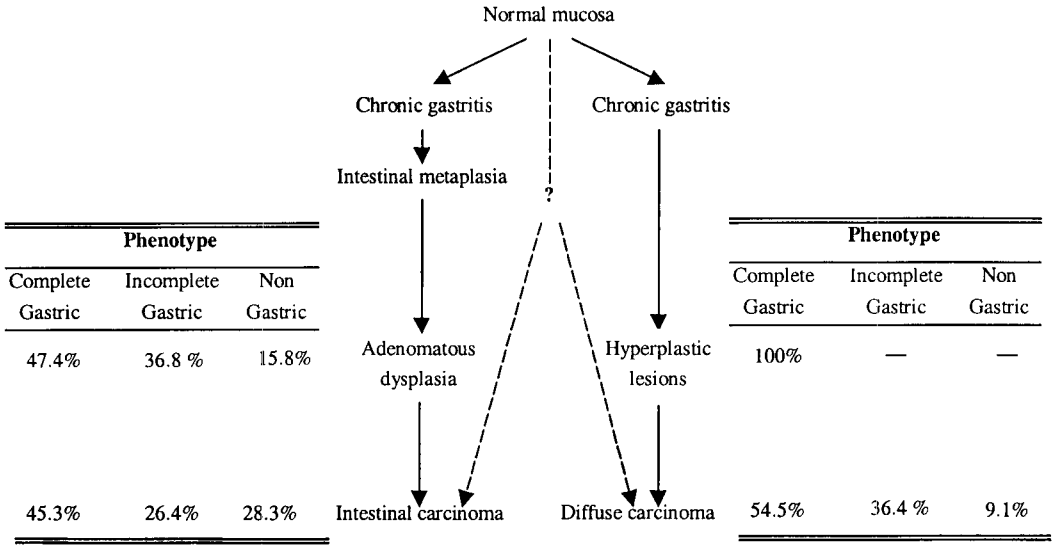
NS, Not significant

Table 13 – Comparison between MUC2 expression and extent of tumour growth of gastric carcinomas according to the histological type.

T stage		MUC2 IHC (%)		
	Cases (%)	+	-	p value
Diffuse				
Early	5 (15.2)	0 (0)	5 (100)	**NS**
Advanced	28 (84.8)	8 (28.6)	20 (71.4)	
Intestinal				
Early	8 (15.1)	1 (12.5)	7 (87.5)	**NS**
Advanced	45 (84.9)	10 (22.2)	35 (77.8)	
Total				**NS**
Early	13 (13.5)	1 (7.7)	12 (92.3)	
Advanced	83 (86.5)	21 (25.3)	62 (74.7)	

NS, Not significant

In figure 8 we summarised the results obtained in the present study, regarding the pattern of cell differentiation along the process of gastric carcinogenesis, having as background the model proposed by Carneiro (1997) and previously depicted in figure 2.



Phenotype				Phenotype		
Complete Gastric	Incomplete Gastric	Non Gastric		Complete Gastric	Incomplete Gastric	Non Gastric
47.4%	36.8 %	15.8%	Adenomatous dysplasia	100%	—	—
45.3%	26.4%	28.3%	Intestinal carcinoma	54.5%	36.4 %	9.1%
			Diffuse carcinoma			

Fig. 8 - Simplified model of gastric carcinogenesis illustrating the two main pathways of malignant transformation, and the prevalences of complete gastric, incomplete gastric and non-gastric phenotypes observed in the different types of pre-malignant and malignant lesions.

Twenty gastric carcinoma cases, classified according to the predominant histological type as intestinal or diffuse carcinomas, displayed small foci with a structure different from the main component of the tumour and were classified as mixed carcinomas. In most cases (n=15), the same cell differentiation pattern was observed in both histological components of the tumours: 11 out of 20 cases (55.0%) were evaluated as complete gastric-type; two cases (10.0%) were classified as incomplete gastric-type; and non-gastric phenotype was observed in two other cases (10.0%).

In five mixed carcinomas (25.0%) we found phenotypic differences between the isolated-cell and glandular components: in two cases complete gastric-type differentiation was observed in the isolated-cell component, whereas in the glandular component we found an expression pattern compatible with incomplete gastric-type differentiation; in one case complete gastric-type differentiation was restricted to the isolated-cell component and non-gastric differentiation was observed in the glandular component; in the remaining two cases, the isolated-cell component of the tumours displayed incomplete gastric phenotype and the glandular component presented non-gastric phenotype.

E-cadherin expression and gene mutations in gastric carcinoma (Papers V and VI).

E-cadherin expression

The pattern of E-cadherin expression was classified as normal whenever the tumours displayed a membranous pattern of staining. All tumours with loss of normal membranous pattern of staining were classified as abnormal, including absence of staining and diffuse cytoplasmic staining.

A normal pattern of E-cadherin expression was observed in 13 out of 50 carcinomas (26.0%) of the whole series. This pattern of immunoreactivity was observed in 11/20 (55.0%) intestinal carcinomas and in 2/28 (7.1%) diffuse carcinomas (Table 14).

An abnormal pattern of E-cadherin staining was observed in 37 out of 50 gastric carcinomas (74.0%). In intestinal carcinomas we observed an abnormal pattern of E-cadherin expression in 9/20 (45.0%) cases and in diffuse carcinomas an abnormal pattern of E-cadherin expression was observed in 26/28 (92.9%). The comparison of the results obtained in the two histotypes yielded statistically significant differences (p=0.0007) (Table 14).

Table 14 - Relationship between the histological type of gastric carcinomas and E-cadherin expression

	E-cadherin IHC			
Histology	**No. of cases (%)**	**Normal (%)**	**Abnormal (%)**	**p value**
Intestinal	20 (40.0)	11 (55.0)	9 (45.0)	
Diffuse	28 (56.0)	2 (7.1)	26 (92.9)	0.0007
Unclassified	2 (4.0)	0 (0.0)	2 (100.0)	
Total	50 (100.0)	13 (26.0)	37 (74.0)	

Eighteen cases were subclassified as mixed carcinomas. In seven of these mixed carcinomas a normal pattern of immunoreactivity was observed in the glandular component and an abnormal pattern was seen in the isolated cell component. In 10 cases

both components of mixed carcinomas displayed an abnormal pattern of immunoreactivity and, in the remaining case, a normal pattern of immunostaining was observed in the two components.

E-cadherin gene mutations

E-cadherin mutations were screened in 26 cases out of the 50 cases included in the whole series. Fifteen mutations of the E-cadherin gene were identified in 12 cases (46.2%); mutations included 10 missense mutations, seven of which occurred in sequences coding for calcium binding motifs, three splice site mutations, one nonsense mutation and one frameshift deletion (Table 15).

In a group of 10 "pure" intestinal carcinomas no mutations were found, and IHC analysis of these cases showed normal E-cadherin expression in eight cases and abnormal expression in two cases (Table 15). Out of 10 "pure" diffuse carcinomas we found mutations in seven cases (70.0%); every diffuse carcinoma harbouring mutations of the E-cadherin gene displayed abnormal E-cadherin expression (Table 15). In two diffuse carcinomas with abnormal expression of E-cadherin (cases 11 and 18) and in the single case of diffuse carcinoma presenting normal E-cadherin expression (case 19) no mutations were detected.

E-cadherin mutations were detected in five of the six cases of mixed gastric carcinoma (83.3%) (Table 15). In four cases we detected mutation of the E-cadherin gene and abnormal E-cadherin expression restricted to the diffuse component of the tumours (cases 21, 23, 24 and 25). In case 26, we found two different mutations in the distinct histological components of the tumour: in the intestinal type component we identified a missense mutation and normal E-cadherin expression; and in the diffuse type component a splice site mutation was associated with abnormal expression of E-cadherin (Table 15). In the single case without detectable E-cadherin mutations, both histological components exhibited normal expression of E-cadherin at the cell membrane (case 22).

A significant relationship was found between the diffuse histotype (both in "pure" and in mixed carcinomas) and E-cadherin mutations ($p<0.0001$). In 16 samples of the diffuse type (10 "pure" diffuse carcinomas and six diffuse components of mixed carcinomas) we found 12 (75.0%) samples presenting E-cadherin mutations. In contrast to this, only one out of the 16 samples of the intestinal type displayed an E-cadherin mutation (conservative missense mutation) and occurring in the intestinal component of a mixed carcinoma.

Loss of heterozygosity analysis.

All cases included in the present study were informative for at least one of the two microsatellite markers analysed. One case out of the 26 (3.8%) showed LOH for marker D16S301. In this case, a missense mutation in one of the calcium binding sequences of the gene was also found (Table 15, case 13). Regarding the DNA polymorphisms detected in this study, the same pattern was observed in tumoural and matched constitutional DNA.

Table 15 - Summary of the results regarding histological type of the cases, E-cadherin mutations and E-cadherin expression pattern in 26 gastric carcinomas.

Case	Histological type	Mutation				IHC
		Exon no.	Codon	Nucleotide change[a]	Predicted protein change	
1	Intestinal	-	-	-	-	Normal
2	Intestinal	-	-	-	-	Normal
3	Intestinal	-	-	-	-	Normal
4	Intestinal	-	-	-	-	Normal
5	Intestinal	-	-	-	-	Normal
6	Intestinal	-	-	-	-	Normal
7	Intestinal	-	-	-	-	Normal
8	Intestinal	-	-	-	-	Abnormal
9	Intestinal	-	-	-	-	Abnormal
10	Intestinal	-	-	-	-	Normal
11	Diffuse	-	-	-	-	Abnormal
12	Diffuse	8	353	1057G→T	E353X (stop)	Abnormal
13	Diffuse	8	370	1108G→C	D370H (missense)	Abnormal
14	Diffuse	10	479	1436A→G	D479G (missense)	Abnormal
15	Diffuse	8	343	1027delC	Frameshift	Abnormal
		9	402	1204G→A	D402N (missense)	
16	Diffuse	12	581	1742T→C	L581P (missense)	Abnormal
17	Diffuse	Intron 7	-	1009-1G→A	In frame deletion	Abnormal
		8	347	1040C→T	A347V (missense)	
18	Diffuse	-	-	-	-	Abnormal
19	Diffuse	-	-	-	-	Normal
20	Diffuse	7	334	1000G→C	D334H (missense)	Abnormal
21	Mixed					
	D	8	369	1105A→G	N369D (missense)	Abnormal
	I	-	-	-	-	Normal
22	Mixed					
	D	-	-	-	-	Normal
	I	-	-	-	-	Normal
23	Mixed					
	D	Intron 7	-	1009-1G→A	In frame deletion	Abnormal
	I	-	-	-	-	Normal
24	Mixed					
	D	9	400	1198G→T	D400Y (missense)	Abnormal
	I	-	-	-	-	Normal
25	Mixed					
	D	8	370	1108G→C	D370H (missense)	Abnormal
	I	-	-	-	-	Normal
26	Mixed					
	D	Intron 7	-	1009-1G→A	In frame deletion	Abnormal
	I	8	365	1093G→C	V365L (missense)	Normal

[a]Numbering is according to the cDNA starting at the A in the start codon (Antonarakis, 1998) (Genbank Accession no. Z13009).

D – Diffuse component of mixed carcinoma.

I – Intestinal component of mixed carcinoma.

IHC - Immunohistochemistry

DISCUSSION

In this section we will discuss our results according to the proposed objectives in the aims. As much as possible, we will try to focus this discussion on the integration of the main results obtained in this study. A detailed discussion of all results is present in Papers I to VI.

Pattern of cell differentiation along the process of gastric carcinogenesis

TFF1 expression

In the first two papers included in this study, we characterised the expression of TFF1 along the process of gastric carcinogenesis. Our findings in normal gastric mucosa confirmed those previously reported by other authors (Rio, *et al.* 1988; Luqmani, *et al.* 1989; Hanby, *et al.* 1993b; Müller and Borchard, 1993): TFF1 expression was detected in the foveolar and superficial epithelium of gastric mucosa, both in the antrum and body/fundus. The expression of TFF1 in gastric epithelium in chronic gastritis was similar to that observed in normal mucosa.

TFF1 immunostaining was found in every case of IM, and we observed that the features of TFF1 expression in metaplastic lesions were specific of IM type; in complete IM, TFF1 expression was restricted to goblet cells and, in types II and III incomplete IM, the staining was observed both in goblet and columnar cells.

These results challenge the sequential evolution of complete to incomplete IM types, along the process of malignant transformation of gastric mucosa, as suggested in the Correa's model of gastric carcinogenesis (Correa, *et al.* 1990a; Correa, *et al.* 1990b; Correa, 1992), which is based mainly upon epidemiological evidence. Although realising the limitations of our study to address such a problem we think we have obtained enough data to claim that in contrast to complete IM, incomplete IM "still" displays features of gastric differentiation, as shown by TFF1 immunostaining in columnar mucus-secreting cells. We feel thus tempted to advance that complete and incomplete types of IM may represent two alternative pathways of metaplastic change in gastric mucosa, rather than two successive steps of phenotypic changes, with complete

IM leading to incomplete IM, as suggested in Correa's model. This hypothesis is supported by the recent results of Reis *et al* (1999), showing that complete IM lacks expression of the gastric mucins MUC5AC and MUC6, while the incomplete forms of IM express MUC5AC in every case and MUC6 in 65.9% of the cases. It remains unclarified, however, if the different IM types represent peculiar responses to different environmental factors or specific adaptations related to physiopathological conditions of the gastric epithelium.

We observed a clear difference between TFF1 expression in hyperplastic and adenomatous polyps, the former displaying a pattern of TFF1 expression which was similar to that observed in normal foveolar and surface epithelium of gastric mucosa. These results fit with the assumption that hyperplastic polyps represent proliferative epithelial lesions which are phenotypically similar to normal gastric epithelium.

At variance with hyperplastic polyps, which display TFF1 immunoreactivity in 100% of the cases, TFF1 was detected in 72.7% of the adenomatous polyps and, in the positive cases, the percentage of immunoreactive cells was significantly lower than in hyperplastic polyps. This down-regulation of TFF1 expression in adenomatous lesions is in keeping with the assumption that adenomatous polyps may serve as precursor lesions of gastric carcinoma of intestinal type, in which we also observed a reduced expression of TFF1.

In gastric carcinomas we found TFF1 expression in about 66% of the cases, and we observed a significantly higher frequency of TFF1 immunoreactivity in diffuse carcinomas than in intestinal carcinomas. These findings concur with those of Theisinger *et al* (1991) and contrast with the results obtained by Müller and Borchard (1993) who did not find any significant relationship between TFF1 expression and the histotype of gastric carcinoma.

Müller and Borchard (1993) found a highly significant correlation between TFF1 immunoreactivity and expression of markers of gastric differentiation, such as pepsinogen II and 2B5, which were co-expressed in most of the TFF1 immunoreactive cells. If we assume, as Müller and Borchard (1993) did, that TFF1 immunoreactivity

discloses the gastric phenotype of neoplastic cells, we may conclude that most diffuse carcinomas display gastric-type differentiation.

These findings are in accordance with ultrastructural data, showing that gastric-type (foveolar and/or mucopeptic) cells are observed in the majority of diffuse carcinomas (Fiocca, *et al.* 1987; Carneiro, *et al.* 1992). Furthermore, about half of the so-called intestinal carcinomas of our series — those displaying TFF1 immunostaining — exhibited signs of gastric-type differentiation. These findings concur with those of Fiocca *et al* (1988a) who showed that, in their series, 55% of the cases of gastric carcinoma with glandular structure expressed pepsinogen II, and with those of Kushima and Hattori (1993) who showed that gastric-type differentiation was present exclusively (23.2%) or in association with intestinal-type differentiation (46.5%) in a series of 43 early gastric carcinomas with glandular structure.

The present and the aforementioned studies show that gastric-type differentiation is present both in diffuse carcinomas and in intestinal carcinomas, though much more often and more expressively in the former than in the latter. The prominence of gastric-type differentiation in diffuse carcinomas suggests that this is the type of carcinoma which is more closely linked to the gastric mucosa both histogenetically and from the differentiation standpoint.

Taking all the above evidence together we think our results fit with the model proposed by Carneiro (1997) (Fig. 2) of at least two pathways of malignant transformation of gastric mucosa: one *via* intestinal metaplasia and adenomatous (flat or polypoid) dysplasia, leading to intestinal carcinomas with intestinal-type differentiation; the other *via* hyperplastic or *de novo* changes, with or without concurrent non-metaplastic dysplasia, leading to diffuse carcinomas and to a subset of intestinal carcinomas with gastric-type differentiation.

Co-expression of trefoil peptides and mucins

In Papers III and IV, we studied the expression of trefoil peptides (TFF1 and TFF2) and mucins (MUC1, MUC2, MUC5AC and MUC6) in premalignant lesions of the stomach

and gastric carcinoma. Our aim was to characterise the expression profile of trefoil peptides and mucins along the process of gastric carcinogenesis, in an attempt to further the understanding of the histogenesis and cell differentiation of gastric carcinoma.. Based on the pattern of expression of trefoil peptides and mucins, we defined three main phenotypes of cell differentiation: complete gastric, incomplete gastric and non-gastric.

In agreement with the conclusions drawn from the study of the expression of TFF1 alone, the results obtained in the analysis of the co-expression of trefoil peptides and mucins showed that hyperplastic polyps displayed a complete gastric-type differentiation. In adenomatous polyps, the pattern of expression of trefoil peptides and mucins was heterogeneous. Complete gastric differentiation was detected in 47.4% of the cases, incomplete gastric differentiation in 36.8% and, in 15.8% of the cases, a non-gastric phenotype was observed.

The present results show that the majority of gastric carcinomas retain signs of complete (52.1%) or incomplete (29.2%) gastric-type differentiation. Such signs are more often exhibited in diffuse and unclassifiable carcinomas than in intestinal-type carcinomas, although gastric-type differentiation was also observed in the latter. In a minority of the cases (18.7%), mostly of the intestinal histotype, we found no signs of gastric-type differentiation.

The comparison of the cell differentiation pattern along the process of gastric carcinogenesis reinforces some of our previous conclusions based on the expression of TFF1 and highlights four points: firstly, the demonstration that intestinal metaplasia of incomplete type displays immunohistochemical markers of gastric differentiation, such as gastric type mucins (Reis, *et al.* 1999) and TFF1 fits with the hypothesis that, in the cascade of events leading to intestinal carcinoma, incomplete intestinal metaplasia (namely type III) (Rokkas, *et al.* 1991; Filipe, *et al.* 1994) may serve as precursor of the adenomatous/dysplastic lesions exhibiting signs of gastric differentiation. Secondly, the three types of cell differentiation are similarly prevalent in the adenomatous lesions and in the intestinal type carcinomas thus supporting the hypothesis that adenomatous/dysplastic lesions do serve as precursors of intestinal-type gastric carcinoma. Thirdly, cell differentiation of gastric type is characteristically retained along

the process of gastric carcinogenesis that leads to diffuse carcinomas, in keeping with the origin of these tumours either *de novo* or from hyperplastic lesions (Carneiro, *et al.* 1993a; Carneiro, *et al.* 1995a). Fourthly, we observed that the absence (loss?) of signs of gastric differentiation occurs in both pathways of gastric carcinogenesis, though more prominently in that leading to intestinal-type carcinomas than that leading to diffuse carcinomas.

The relationship between the pattern of cell differentiation and the histological type of the tumours, is further supported by our findings in mixed gastric carcinoma. In cases where the two histological components of the tumours presented a different pattern of cell differentiation, the isolated-cell component always displayed a more conserved gastric-type differentiation. Our finding that most mixed carcinomas keep a similar cell differentiation pattern in both histological components supports the assumption that both components of these tumours derive from the same parental neoplasia.

MUC2 is characteristically expressed in goblet cells of native intestinal epithelium (Ho, *et al.* 1993; Ho, *et al.* 1995; Reis, *et al.* 1998). In the present study, MUC2 was expressed in relatively few cases (22.9%). We found no significant relationship between the expression of this molecule and the histotype and cell differentiation pattern of the tumours, in keeping with data previously reported (Filipe, *et al.* 1996; Reis, *et al.* 1998). The expression of MUC2 in carcinomas displaying a gastric phenotype may indicate the existence of a mixed differentiation (gastric and intestinal) in these tumours; this possibility remains however disputable because we observed a low prevalence of MUC2 expression in the group of tumours that in principle should depict the highest degree of intestinal differentiation: carcinomas of the intestinal histotype with a non-gastric phenotype.

Regarding the relationship between cell differentiation pattern and tumour growth, we observed that absence (loss?) of native gastric differentiation appears to be related with tumour progression, increasing from early to advanced carcinomas, both in intestinal and diffuse carcinomas. Ultrastructural and immunohistochemical data on record (Fiocca, *et al.* 1987; Yamachika, *et al.* 1997) led to the suggestion that there is acquisition of signs of intestinal differentiation along tumour progression. Our own

results show that MUC2 expression increases from early (7.7%) to advanced (25.3%) carcinomas, though the differences did not attain the threshold of statistical significance. Putting together our findings and those previously reported we can not exclude that, at least in some cases, absence/loss of gastric differentiation may be paralleled by intestinalisation of gastric carcinomas.

Altogether, our data suggest that a large proportion of gastric carcinomas, regardless of histotype, derive from native gastric epithelium and retain signs of gastric differentiation. Whether these carcinomas arise *de novo* or from hyperplastic/dysplastic lesions remains to be fully elucidated. The expression of MUC2 mucin both in intestinal and diffuse carcinomas is in keeping with data on record pointing to the existence of signs of intestinal differentiation in both types of carcinomas (Tatematsu, *et al.* 1986; Fiocca, *et al.* 1987; Fiocca, *et al.* 1988b; Carneiro, *et al.* 1992; Kushima, *et al.* 1993; Yamachika, *et al.* 1997). It remains to be seen if the signs of intestinal differentiation are related with the precursor lesions of these tumours or, otherwise, reflect a phenomenon of intestinalisation along tumour progression, as previously described in the literature (Fiocca, *et al.* 1987; Yamachika, *et al.* 1997). The former being true, highlights the possibility that at least some diffuse carcinomas may also originate from the histogenetic pathway leading to intestinal carcinomas with mixed- or intestinal-type differentiation.

Role of E-cadherin in morphologic heterogeneity of gastric carcinoma

In the present study, we found abnormal E-cadherin expression in 74.0% of gastric carcinomas, a prevalence that is close to that reported by other authors (Yonemura, *et al.* 1995; Gabbert, *et al.* 1996). Furthermore, we found a significant higher frequency of abnormal E-cadherin expression in diffuse carcinomas (92.9%) than in intestinal carcinomas (45.0%). This finding is in agreement with those reported previously, pointing to a correlation between altered expression of E-cadherin and either diffuse or poorly differentiated/undifferentiated gastric carcinoma (Mayer, *et al.* 1993; Shino, *et al.* 1995; Gabbert, *et al.* 1996; Jawhari, *et al.* 1997).

The separate evaluation of E-cadherin expression in distinct histological components of the mixed carcinomas consistently reproduced the association between E-cadherin expression pattern and the histotype. E-cadherin expression was scored as abnormal in the diffuse/isolated cell-type component in 17/18 (94.4%) mixed carcinomas. At variance with this, in the intestinal/glandular component of mixed carcinomas, abnormal E-cadherin expression was observed in 10/18 (55.6%) cases, the remaining eight cases (44.4%) exhibiting, in the glandular areas, a normal (polarised) pattern of E-cadherin expression at the cell membrane.

This significant relationship between an abnormal expression of E-cadherin and diffuse carcinomas, which is kept both in "pure" carcinomas and in the isolated-cell component of mixed carcinomas, is in keeping with the hypothesis that E-cadherin plays a crucial role in the adhesion among neoplastic cells which, if disrupted, leads to dissociation of cells and scattered growth.

This hypothesis is further supported by our results on the mutational analysis of the E-cadherin gene. We detected E-cadherin gene mutations in 46.2% of gastric carcinomas and by sequencing analysis we identified 15 mutations in 12 cases, mostly of the missense type.

In the classical two hit model of tumour suppressor gene inactivation, mutations in one allele are accompanied by deletion of the remaining normal allele. Our results appear to challenge this model since LOH of 16q22.1 was found in only one of the 12 cases presenting mutation of the E-cadherin gene. Noteworthy, in case 15 two different inactivating mutations were identified thus fulfilling also the two hit inactivation model. These findings contrast with those of Berx et al (1996) in lobular carcinoma of the breast, showing that 93% of cases with E-cadherin gene mutation displayed also LOH. Alternative inactivation mechanisms of the remaining normal allele may include specific allelic exclusion (Becker and Hofler, 1995) or alterations at the gene promoter level (Hennig, *et al.* 1995). Methylation within the promoter or the 5'-CpG island of the E-cadherin gene has been shown in breast and prostate carcinomas (Graff, *et al.* 1995; Hennig, *et al.* 1995; Yoshiura, *et al.* 1995). Additionally, one can speculate that the mutation of one allele may result in a dominant-negative effect over the remaining

normal allele. Cadherins exist at the cell surface as dimers linked by interactions at each cadherin domain (Shapiro, *et al.* 1995). One may admit that a mutated E-cadherin protein may still bind to wild-type protein leading to a non-functional E-cadherin complex.

Although we observed a very good correlation between abnormal E-cadherin expression and gene mutations it is not possible to establish a linear relationship between the type of mutation and the expression patterns of the protein. While the occurrence of mutations in the E-cadherin gene seems to be a critical step in diffuse gastric carcinogenesis and remains fixed throughout progression of the tumour, the expression pattern at the moment of analysis is the result of both mutation in the E-cadherin gene and the specific "tumour-environment" interplay, which involves an altered balance of transcription factors, expression and repression of many genes and frequent structural modifications at the DNA level. Regardless of the type of mutation, abnormal E-cadherin expression was mainly characterised by cytoplasmic expression and, in some cases, by a mixture of cytoplasmic expression and lack of expression. We may speculate that the internalisation of a non-functional E-cadherin complex (Kartenbeck, *et al.* 1991) or deficient transport of an abnormal E-cadherin protein to the cell membrane could explain the cytoplasmic immunoreactivity.

In the present series, E-cadherin mutations occurred in 70% of "pure" diffuse gastric carcinomas and were not detected in "pure" intestinal carcinomas. This very strong association between E-cadherin mutations and the diffuse histotype is in agreement with the results of Becker *et al* (1994), who reported mutations of the E-cadherin gene in 50% of diffuse gastric carcinomas and their absence in intestinal type carcinomas. Furthermore, E-cadherin mutations were detected in gastric carcinoma cell lines with a diffuse phenotype (Oda, *et al.* 1994) and in families of patients with diffuse gastric carcinoma (Gayther, *et al.* 1998; Guilford, *et al.* 1998; Richards, *et al.* 1999). In mixed gastric carcinomas, we observed that inactivating E-cadherin mutations were restricted to the diffuse component of the tumours in five out of six cases. This finding suggests the existence of genotypically divergent tumour clones in mixed gastric carcinoma and

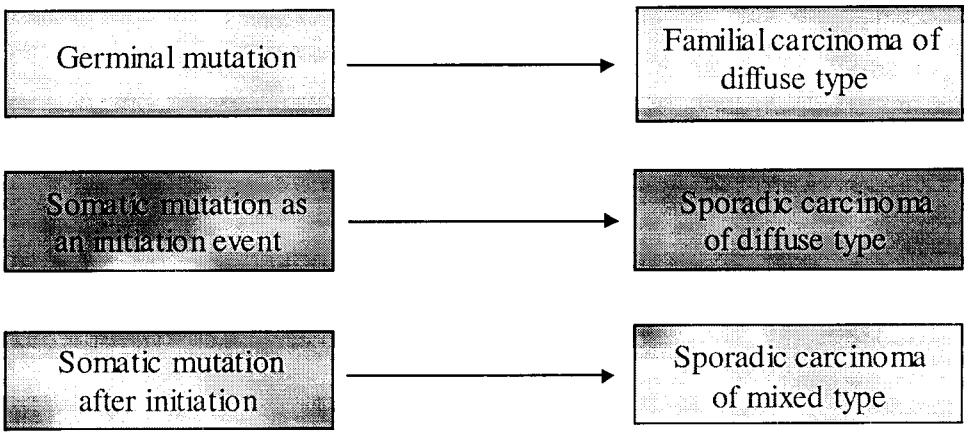
indicates that E-cadherin inactivation is the hallmark of the dual morphologic divergence in this type of tumours.

Altogether, these results suggest that inactivation of the E-cadherin gene determines the diffuse histotype of gastric carcinoma. This putative cause-effect relationship between E-cadherin inactivation and the diffuse histotype is challenged by the existence of a small percentage of cases presenting diffuse histotype without detectable E-cadherin mutation and, in two cases (case19 and diffuse component of case 22), even displaying normal protein expression. In such cases, one has to admit that the adhesion of neoplastic cells is disrupted by an alteration at another level of the cell adhesion system. As pointed out in the introductory section of this Thesis, E-cadherin is only one of the constituents of a complex cell adhesion system and its function is dependent on the interaction of its conserved cytoplasmic domain (C-terminal) with the cytoskeleton *via* molecular complexes involving α-, β- and γ-catenin (Kemler, 1993). Alterations in the associated cytoplasmic molecules, such as the catenins, are thought to impair cell adhesion in tumours exhibiting normal E-cadherin expression (Shimoyama, *et al.* 1992; Jawhari, *et al.* 1997; Smith and Pignatelli, 1997; Bukholm, *et al.* 1998).

The significant relationship between E-cadherin mutation and the diffuse histotype of gastric carcinoma and the restriction of E-cadherin inactivating mutations to the diffuse component of mixed carcinomas, suggest that the timing of E-cadherin inactivation and the timing of phenotypic divergence probably coincide in mixed gastric carcinomas. The findings of Muta *et al* (1996) and Vos *et al* (1997), showing E-cadherin alterations in intramucosal diffuse gastric carcinoma and in *in situ* lobular carcinoma of the breast, respectively, indicate that E-cadherin inactivation is an early event in carcinogenesis. Moreover, it has been recently shown that loss of E-cadherin expression occurs in the transition from adenoma to carcinoma in a transgenic mouse model of pancreatic β-cell carcinogenesis (Perl, *et al.* 1998). Based on these findings it is tempting to suggest that phenotypic divergence of mixed gastric carcinoma occurs early during neoplastic development. This assumption is indirectly supported by the presence of E-cadherin mutations in patients with familial diffuse gastric carcinoma (Gayther, *et al.* 1998;

Guilford, *et al.* 1998; Richards, *et al.* 1999) indicating that E-cadherin inactivation may even be an initiation event in gastric carcinogenesis.

Putting together data we obtained and that on record we feel tempted to suggest that the involvement of the E-cadherin gene in gastric carcinogenesis can be summarised as follows:



Histogenetic pathways of gastric carcinogenesis revisited

The results obtained in this study show that the majority of gastric carcinomas retain signs of gastric-type differentiation, more often exhibited in diffuse than in intestinal carcinomas. These findings support our contention that carcinomas forming glands should be designated as glandular carcinomas rather than intestinal carcinomas in order to avoid mixing structural with cell differentiation concepts. Non-gastric phenotype occurs also in both types of gastric carcinoma, though more prominently in intestinal carcinoma. Furthermore, we observed that loss of native gastric differentiation is related with tumour progression, increasing from early to advanced carcinomas, both in intestinal and diffuse carcinomas.

The comparison of the patterns of cell differentiation between premalignant lesions and gastric carcinomas shows good agreement with the model proposed by Carneiro (1997)

and supports that there are at least two pathways of malignant transformation of gastric mucosa: one *via* intestinal metaplasia and adenomatous (flat or polypoid) dysplasia, leading to intestinal-type carcinomas; the other *via* hyperplastic or *de novo* changes, with or without concurrent non-metaplastic dysplasia, leading to diffuse carcinomas and to a subset of intestinal carcinomas with gastric-type differentiation (Fig. 2).

Our results on the expression of MUC2 in gastric carcinoma and data on the literature (Tatematsu, *et al.* 1986; Fiocca, *et al.* 1987; Fiocca, *et al.* 1988b; Carneiro, *et al.* 1992; Kushima, *et al.* 1993; Yamachika, *et al.* 1997) show that intestinal-type differentiation may occur both in intestinal and diffuse carcinoma. It remains to be seen if MUC2 expression reflects a histogenetic pathway related with intestinal metaplasia or, otherwise, represents *de novo* expression along tumour progression.

Together with the data provided by other authors on E-cadherin inactivation in sporadic (Becker, *et al.* 1993; Becker, *et al.* 1994) and familial (Gayther, *et al.* 1998; Guilford, *et al.* 1998; Richards, *et al.* 1999) gastric carcinoma, our results strongly support that E-cadherin inactivation (or, more generally, loss of cell-cell adhesion) determines the diffuse histotype. Furthermore, we have shown that mixed gastric carcinomas originate most probably from intestinal-type carcinomas through loss of E-cadherin function, leading to the establishment of an isolated-cell histological component.

Accepting this hypothesis as valid, the study of trefoil peptides and mucins suggests that the majority of mixed carcinomas may originate from intestinal carcinomas with gastric phenotype. Loss of gastric differentiation in the intestinal component of some mixed carcinomas that display signs of gastric differentiation in the diffuse component remains to be explained.

Gastric carcinogenesis is a multistage process in which successive rounds of mutation and clonal selection in tumour cells underlie the progression of the neoplasia. If we extrapolate this reality to the model of mixed carcinoma, one may admit that in the natural history of this type of tumour one of the histological components may overgrow the other. Despite this possibility, it seems that the two histological components of mixed carcinomas may coexist and, perhaps, even co-operate, providing a putative

explanation for the biological aggressiveness of mixed carcinoma reflected in the poor survival of the patients (Carneiro, *et al.* 1995b; Carneiro, 1997).

This hypothesis implies that mixed gastric carcinoma is one, eventually the major, player in the generation of gastric carcinoma diversity, providing theoretical ground for the structural and cell differentiation heterogeneity observed in carcinoma of the stomach.

Based on the aforementioned evidence and on our personal interpretation of the data, we propose the model depicted in figure 9 as a backbone to explain the morphologic and cell differentiation heterogeneity associated with gastric carcinoma. Among the many limitations that this model certainly presents, we would like to stress the following two: firstly, we can still not exclude the possibility that gastric carcinoma arises *de novo* from normal gastric epithelium; secondly, the histogenesis of unclassifiable/solid carcinomas is still an enigma, i.e. it is not known if it represents the end-stage of one and/or two of the major types of gastric carcinoma or if it is the result of an independent pathway of carcinogenesis.

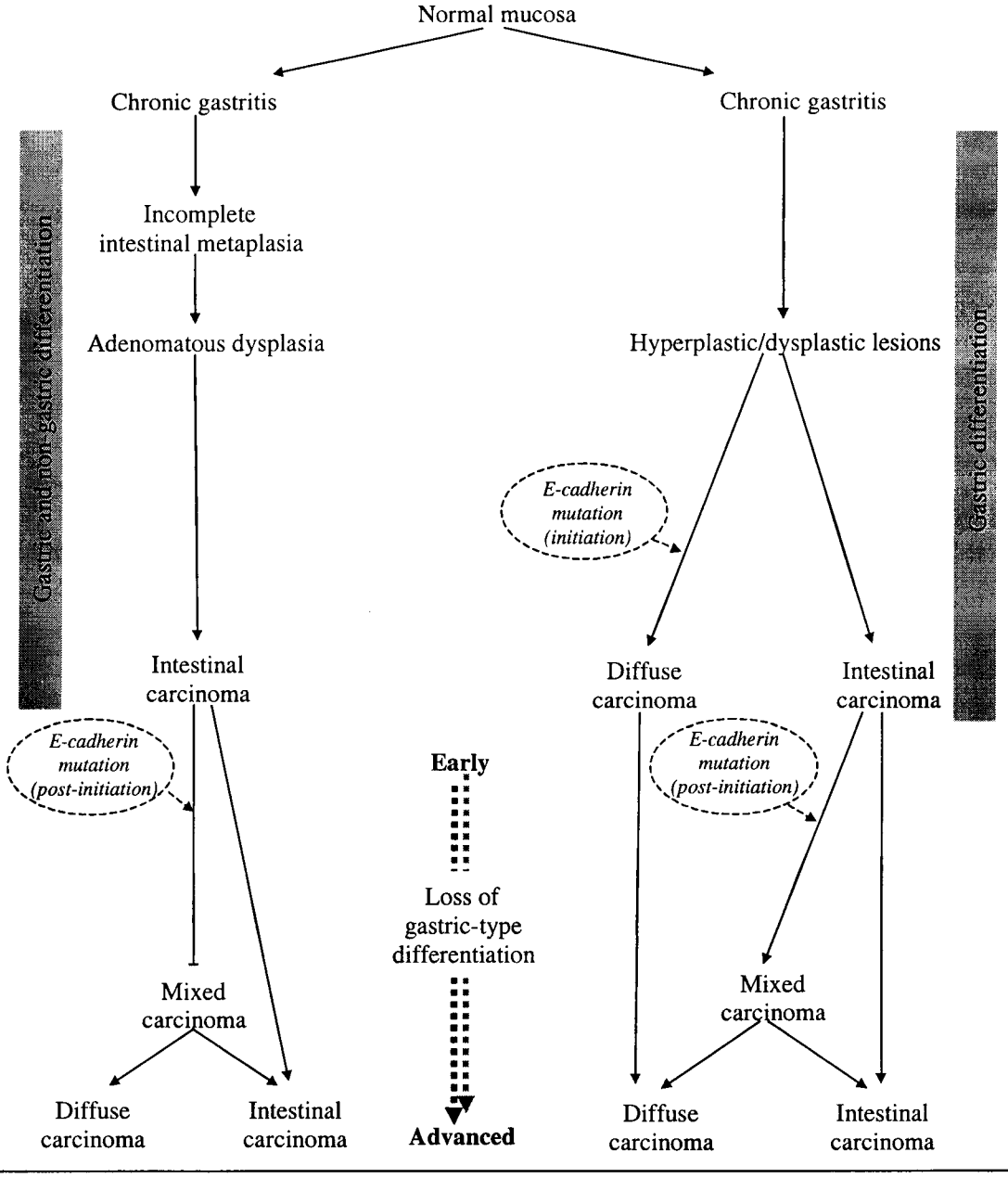


Fig. 9 – Model of gastric carcinogenesis illustrating the two main histogenetic pathways and the central role of mixed carcinoma in the generation of morphologic and cell differentiation heterogeneity.

Future perspectives

We believe that the results of this study will help to further the understanding of the biopathogenesis of gastric carcinoma. Nevertheless, many problems remain unsolved and our own results raise some additional questions.

An increasing amount of evidence is being produced in support of the hypothesis that trefoil peptides act as motogenic factors in the process of restitution of gastrointestinal epithelia. Most probably, this effect occurs through modulation of the E-cadherin-mediated cell-cell adhesion system. The elucidation of this putative inter-relation between trefoil peptides and E-cadherin constitutes a very promising research field.

Although we and other authors have shown that E-cadherin inactivation appears to be one of the key events underlying both initiation and/or progression of a large part of diffuse gastric carcinomas, a proportion of these type of tumours, including sporadic and familial forms, keep an apparently normal E-cadherin. In the discussion chapter of the Thesis we have advanced that in these cases other components of the E-cadherin cell-cell adhesion system may be inactivated rendering the normal E-cadherin protein useless for the adhesion process. This is an aspect that needs further investigation.

One of the most interesting findings of our results is the fact that E-cadherin inactivation seems to underlie the histological divergence of mixed gastric carcinoma that gives rise to two individual morphologic components. In order to fully elucidate this finding one needs to confirm the monoclonal origin of these type of tumours. Furthermore, the study of additional genetic alterations which may, or may not, be shared by the two histological components of mixed carcinomas will further our understanding of the biopathology of this particular type of tumour.

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SUMÁRIO E CONCLUSÕES

O carcinoma do estômago continua a constituir uma das principais causas de morte por doença oncológica em Portugal. A incidência de carcinoma gástrico em Portugal é a mais elevada da União Europeia e, contrariamente ao verificado na maior parte dos países ocidentais, não se tem assistido, entre nós, a um declínio significativo das taxas de incidência e mortalidade por este tipo de neoplasia.

É geralmente aceite a existência de duas vias principais de transformação maligna da mucosa gástrica: uma através da metaplasia intestinal e da displasia adenomatosa, levando à formação de carcinomas do tipo intestinal; a outra através de alterações hiperplásicas/displásicas originando carcinomas do tipo difuso e um subgrupo de carcinomas do tipo intestinal.

A diversidade morfológica do carcinoma gástrico é muito maior do que a esperada tendo em conta a existência de dois tipos histológicos principais — intestinal e difuso. De facto, uma grande parte dos carcinomas gástricos são polimórficos e exibem grande diversidade tanto estrutural como de diferenciação celular. Do ponto de vista molecular, as alterações subjacentes à heterogeneidade do carcinoma gástrico estão longe de estar esclarecidas.

No presente trabalho seleccionámos os "trefoil peptides" (e a sua co-expressão com mucinas) e a caderina-E como "instrumentos" para estudar alguns aspectos de diferenciação celular e de diversidade estrutural do carcinoma do estômago. Foi nosso objectivo analisar os seguintes aspectos da biopatologia do carcinoma gástrico: 1-) padrão de diferenciação celular do carcinoma gástrico e de lesões pré-malignas do estômago; 2-) mecanismos moleculares subjacentes à divergência estrutural do carcinoma do estômago, reflectida na existência de dois tipos principais de carcinoma — intestinal e difuso; 3-) características fenotípicas e moleculares associadas ao carcinoma gástrico de tipo misto; 4-) contribuição dos resultados dos diferentes estudos para a clarificação da histogénese do carcinoma gástrico.

Apresentam-se resumidamente os resultados de cada um destes estudos:

1 – Padrão de diferenciação celular do carcinoma gástrico e de lesões pré-malignas do estômago.

Dados da literatura relativos à caracterização ultrastrutural, histoquímica e imunohistoquímica das neoplasias permitiram verificar que o carcinoma gástrico exibe, de forma isolada ou em combinação, dois tipos principais de diferenciação celular — gástrica e intestinal.

Nesta parte do estudo caracterizámos os padrões de diferenciação celular no carcinoma gástrico e em lesões pré-malignas do estômago, incluindo gastrite crónica atrófica, metaplasia intestinal e pólipos gástricos (de tipo hiperplásico e adenomatoso).

Para a prossecução deste objectivo procedemos à avaliação dos padrões de expressão de "trefoil peptides" específicos do estômago (TFF1 e TFF2) em lesões pré-malignas e no carcinoma gástrico. Paralelamente, analisámos a co-expressão destes "trefoil peptides" com mucinas — MUC5AC e MUC6 (mucinas gástricas), MUC2 (mucina intestinal) e MUC1.

Os resultados obtidos neste estudo mostraram que a maioria dos carcinomas gástricos retêm sinais de diferenciação gástrica, mais frequentemente expressa nos carcinomas de tipo difuso do que nos carcinomas de tipo intestinal. A ausência de diferenciação gástrica (fenótipo não-gástrico) foi observada numa pequena percentagem de casos de ambos os tipos histológicos principais de carcinoma do estômago, sendo mais notória no carcinoma intestinal. Verificámos ainda uma tendência para a perda de diferenciação gástrica ao longo da progressão do carcinoma gástrico, mais acentuada no carcinoma intestinal.

Detectámos a expressão de MUC2 (mucina intestinal) em pequena percentagem de carcinomas gástricos. Observámos ainda uma tendência para o aumento de expressão de MUC2 em carcinomas avançados. No entanto, os nossos resultados não permitem esclarecer de forma conclusiva se a expressão de MUC2 reflecte uma histogénese relacionada com a metaplasia intestinal (onde a mucina MUC2 é expressa normalmente) ou, pelo contrário, representa uma expressão *de novo* relacionada com a progressão tumoral (traduzindo a designada "intestinalização" do carcinoma gástrico).

2 – Mecanismos moleculares subjacentes à divergência estrutural do carcinoma do estômago.

Estudámos a expressão da caderina-E no carcinoma gástrico e analisámos as alterações do respectivo gene subjacentes às alterações de expressão da proteína. Encontrámos uma associação significativa entre a expressão anormal (ausente e/ou citoplasmática) da caderina-E e o carcinoma gástrico difuso. Através da pesquisa de mutações no gene da caderina-E verificámos a ocorrência de diversos tipos de mutações ("missense", "non-sense", "frameshift deletion" e "splice site") na maior parte (70%) dos carcinomas difusos e a sua ausência nos carcinomas de tipo intestinal. Estes resultados, concordantes com os da literatura, indicam que a inactivação da caderina-E é determinante para a definição do fenótipo de tipo difuso do carcinoma gástrico.

3 – Características fenotípicas e moleculares associadas ao carcinoma gástrico de tipo misto.

A heterogeneidade morfológica do carcinoma gástrico reflecte-se também nos casos individuais; muitos carcinomas gástricos são pluriformes, exibindo dois ou mais componentes histológicos distintos. A maioria das classificações histológicas ignoram este aspecto, agrupando os carcinomas gástricos de acordo com o componente histológico predominante. Este facto tem dificultado o esclarecimento da biopatologia do carcinoma gástrico de tipo misto.

Neste estudo decidimos analisar individualmente os diferentes componentes do carcinoma misto relativamente à diferenciação celular e à existência de alterações estruturais e/ou de expressão da caderina-E.

Verificámos que, na maior parte dos carcinomas mistos, o padrão de diferenciação celular é o mesmo em ambos os componentes histológicos do tumor e é predominantemente de tipo gástrico. Pesquisámos mutações do gene da caderina-E, estudando separadamente os diferentes componentes das neoplasias através de microdissecção dos tumores, e observámos a ocorrência de mutações do gene da caderina-E apenas no componente difuso das neoplasias. A única mutação que

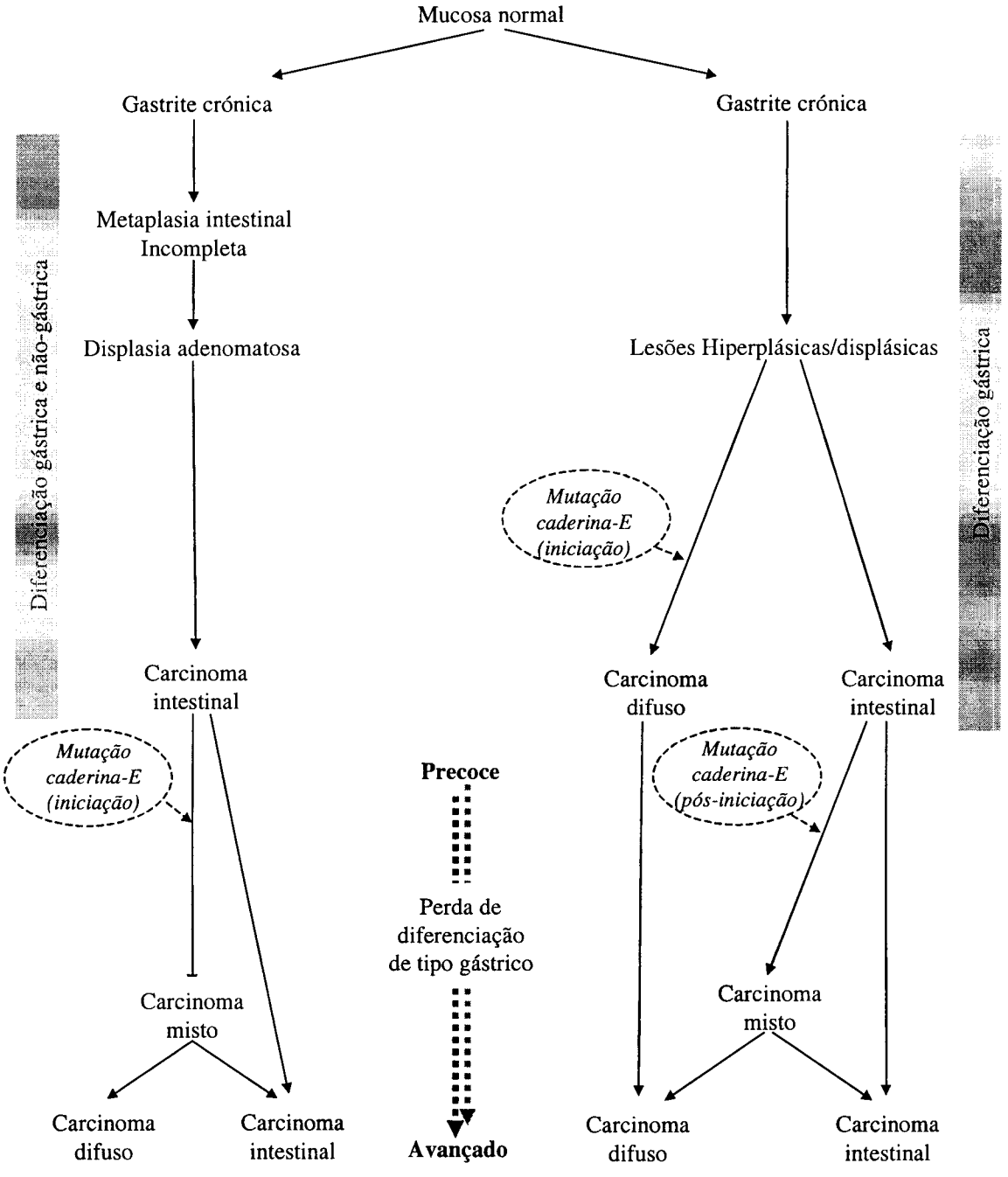
identificámos no componente glandular de tumores mistos foi uma mutação silenciosa, sem consequências funcionais para a proteína codificada.

Estes resultados permitiram-nos sugerir, pela primeira vez na literatura, a existência de uma base genética para a divergência fenotípica do carcinoma gástrico. Estes resultados, aliados aos obtidos no estudo das alterações da caderina-E em carcinomas gástricos "puros", levam-nos a sugerir que o envolvimento da caderina-E na carcinogénese gástrica de tipo esporádico é de dois tipos; mutações somáticas coincidentes com a iniciação neoplásica e associadas ao carcinoma difuso; mutações somáticas após a iniciação neoplásica, associadas ao carcinoma de tipo misto.

4 – Contribuição dos resultados dos diferentes estudos para a clarificação da histogénese do carcinoma gástrico.

O nosso objectivo último foi o de integrar a informação derivada dos estudos previamente descritos na tentativa de contribuir para a clarificação das vias histogenéticas conducentes ao carcinoma gástrico de tipo intestinal, difuso e misto.

Com base nos resultados que obtivemos, propomos o modelo representado na figura seguinte para explicar a heterogeneidade morfológica e de diferenciação celular do carcinoma gástrico, no qual o carcinoma gástrico de tipo misto desempenha um papel central.


Mucosa normal
Gastrite crónica
Gastrite crónica
Metaplasia intestinal Incompleta
Displasia adenomatosa
Lesões Hiperplásicas/displásicas
Diferenciação gástrica e não-gástrica
Diferenciação gástrica
Mutação caderina-E (iniciação)
Carcinoma intestinal
Carcinoma difuso
Carcinoma intestinal
Mutação caderina-E (iniciação)
Mutação caderina-E (pós-iniciação)
Precoce
Perda de diferenciação de tipo gástrico
Carcinoma misto
Carcinoma misto
Carcinoma difuso
Carcinoma intestinal
Avançado
Carcinoma difuso
Carcinoma intestinal

PAPER I

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Pattern of pS2 protein expression in premalignant and malignant lesions of gastric mucosa

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The aim of this study was to evaluate the pattern of pS2 protein expression in premalignant and malignant lesions of gastric epithelium. We analysed, by immunohistochemistry, the pS2 expression in six samples of normal gastric mucosa, 18 cases of chronic atrophic gastritis with intestinal metaplasia (IM), 10 hyperplastic polyps, 11 adenomatous polyps and 50 gastric carcinomas, together with the respective samples of adjacent non-neoplastic mucosa. pS2 is expressed throughout foveolar and superficial epithelium of normal gastric mucosa and this pattern is retained in chronic atrophic gastritis out of IM lesions. pS2 expression is confined to goblet cells in complete IM and occurs both in goblet and columnar cells in incomplete IM. Hyperplastic polyps displayed significantly higher pS2 expression than adenomatous polyps. In gastric carcinomas, pS2 expression was observed in 66.0% of cases, being significantly higher in diffuse (88.9%) than intestinal type carcinomas (53.6%). A subset of carcinomas of the latter group displayed pS2 immunoreactivity in a high percentage of cells with a pattern similar to that of hyperplastic polyps. Our results demonstrate there are major changes in pS2 expression, which can be used as a marker of gastric-type differentiation during the process of gastric carcinogenesis, and support the existence of at least two pathways of malignant transformation of gastric mucosa: one via intestinal metaplasia and adenomatous dysplasia, leading to glandular carcinomas with intestinal-type differentiation, the other via hyperplastic changes or *de novo* changes, leading to diffuse carcinomas and to a subset of glandular carcinomas displaying gastric-type differentiation.

Key words: Chronic atrophic gastritis, gastric carcinoma, gastric polyps, immunohistochemistry, intestinal metaplasia, mucins, pS2, stomach, trefoil peptides.

Introduction

Gastric carcinoma is very heterogeneous from the morphologic standpoint, probably reflecting the complexity and diversity of the process(es) of malignant transformation of gastric mucosa (Laurén 1965; Correa 1992; Carneiro *et al*, 1995b). According to Laurén's classification, there are two main types of gastric carcinoma—intestinal and diffuse—which differ from each other epidemiologically, morphologically and clinically (Laurén 1965; Carneiro *et al*, 1995b).

Correa proposed a model of gastric carcinogenesis, according to which the so-called intestinal carcinoma represents the end-product of a cascade of sequential changes of gastric mucosa including superficial gastritis, chronic atrophic gastritis, small intestinal metaplasia, colonic metaplasia and adenomatous (flat or polypoid) dysplasia (Correa 1988, 1992).

Correa's proposal found strong support in many morphologic and epidemiologic studies (Correa *et al*, 1990a,b; Filipe *et al*, 1994). However, this model does

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not fit with a subset of glandular carcinomas (classified as intestinal carcinomas according to Laurén's classification) that show histochemical signs of gastric-type differentiation and no evidence of intestinal-type differentiation. These gastric-type adenocarcinomas were thought to originate from foveolar hyperplasia rather than from intestinal metaplasia (IM) Hattori 1985; Kushima and Hattori, 1993).

From the histogenetic standpoint it is generally accepted that diffuse carcinomas originate from gastric proper mucosa (Ghandur-Mnaymneh *et al*, 1988) and that non-metaplastic dysplasia is a putative premalignant lesion of this type of carcinoma (Ghandur-Mnaymneh *et al*, 1988), although little is known regarding the prevalence and clinicopathologic features of the aforementioned form of dysplasia. Furthermore, there is growing evidence that hyperplastic lesions of gastric mucosa (foveolar hyperplasia and hyperplastic polyps) may also serve as precursor lesions of the diffuse type of gastric carcinoma (Carneiro *et al*, 1993a,b; Zea-Iriarte *et al*, 1995).

pS2 protein is a member of the trefoil peptide family whose gene was originally cloned in MCF-7 breast cancer cell line (Masiakowski *et al*, 1982). The ultimate function of trefoil peptides is presently unclear, but recent data suggest that they may play a major role in the mechanisms of protection and regeneration of epithelia of the gastrointestinal tract (Wright *et al*, 1990; Rio *et al*, 1991; Seitz *et al*, 1991; Hanby *et al*, 1993b,c; Alison *et al*, 1995).

In normal human tissues pS2 immunoexpression has only been consistently reported in stomach (Rio *et al*, 1988; Piggott *et al*, 1991), where it decorates the superficial and foveolar epithelium (Rio *et al*, 1988; Luqmani *et al*, 1989; Wright *et al*, 1990; Hanby *et al*, 1993b; Müller and Borchard, 1993). Apart from a very brief description of pS2 expression in superficial gastritis, atrophic gastritis and dysplasia by Luqmani *et al* (1989), there are no reports on the pattern of pS2 expression in the so-called premalignant lesions of gastric mucosa on record.

The few reports on the pS2 immunoexpression in gastric carcinoma (Luqmani *et al*, 1989; Henry *et al*, 1991; Theisinger *et al*, 1991; Müller and Borchard, 1993) show a tendency towards the loss of pS2 immunoexpression in carcinomas in comparison to its expression in normal epithelium. Theisinger *et al* (1991) observed, moreover, an association between retention of pS2 expression and diffuse type of gastric carcinoma, but this finding was not confirmed in the series of Müller and Borchard (1993).

In the present study we evaluated the immunohistochemical expression of pS2 in six samples of normal gastric mucosa, 18 cases of chronic atrophic gastritis with IM, 21 gastric polyps (10 hyperplastic polyps and 11 adenomatous polyps) and 50 gastric carcinomas, together with the respective samples of adjacent non-neoplastic mucosa, in an attempt to analyse the changes of pS2 expression along the process of gastric carcinogenesis.

Material and methods

Tissue material

Six cases with normal gastric biopsies and 18 cases of chronic atrophic gastritis with IM, all of them from dyspeptic patients without gastric carcinoma or other focal lesions at endoscopy, were included in the study. From each patient there were six biopsies available (two from the antrum, two from the incisura angularis and two from the body/fundus). Twenty-one gastric polyps and surgical specimens from 50 gastric carcinomas consecutively resected at Hospital S. João-Medical Faculty of Porto were also studied.

The tissue fragments were fixed in 10% formaldehyde and embedded in paraffin. Serial sections of 4 μm were cut from each block and used for routine staining with haematoxylin and eosin, histochemical study with periodic acid–Schiff (PAS), alcian blue pH 2.5 (AB)/PAS and AB/high iron diamine (HID), and immunohistochemistry.

Histological and histochemical classifications

Gastritis was classified according to Whitehead (1985). In the presence of glandular atrophy, gastritis was considered as chronic atrophic gastritis, regardless of the co-existence of IM. IM was classified according to Filipe and Jass (1986) into types I, II and III in sections stained with AB/PAS and HID/AB. Gastric polyps were classified according to Whitehead (1985) into hyperplastic polyps (n = 10) and adenomatous polyps (n = 11). Gastric carcinomas were classified according to Laurén (1965) as intestinal (n = 28), diffuse (n = 18) and unclassifiable carcinomas (n = 4).

Immunohistochemistry

Monoclonal antibody (MoAb) BC4 (CIS Bio International, Gif-Sur-Yvette, France) was used for the immunohistochemical study of the expression of pS2 in formalin-fixed paraffin-embedded tissues. A modification of the avidin–biotin–peroxidase complex method (Hsu *et al*, 1981) was applied. The paraffin

sections (4 μm thick) were dewaxed, incubated for 30 min at 37°C in a 0.01 M HCl solution containing 0.4% pepsin and then rinsed in TRIS-buffered saline (TBS), pH 7.6. The sections were treated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase, washed in TBS, then incubated for 20 min with normal rabbit serum, at a dilution of 1:5 in TBS containing 25% bovine serum albumin (BSA). Excess normal serum was removed and replaced by the MoAb BC4 diluted at 1:4. After overnight incubation (18 hours) at 4°C, slides were washed with TBS, and the sections incubated with a 1:200 dilution of biotin-labelled secondary antibody, for 30 min. After washing with TBS, sections were incubated with avidin–biotin–peroxidase complex (10 mg/ml of biotin-labelled peroxidase) for 60 min. This was followed by staining the sections for 5 min with 0.05% diaminobenzidine, freshly prepared in 0.05 M TRIS buffer, pH 7.6, containing 0.01% hydrogen peroxide. Finally, sections were counterstained with haematoxylin, dehydrated and mounted. Dilution of primary antibodies, biotin-labelled secondary antibodies and avidin–biotin–peroxidase complex were made with TBS containing 12.5% BSA.

All series included normal gastric mucosa as positive controls. Negative controls were performed by substitution of the primary antibody with IgG_1 immunoglobulins of the same class and concentration as the MoAb.

Scoring

A case was considered positive whenever more than 5% of the cells displayed histochemical or immunohistochemical staining, irrespective of the localization of the positive cells and the intensity of the staining. Whenever possible, the cell localization of the staining was classified as perinuclear (Golgi area), cytoplasmic (diffuse or apical) or membranous (at the cell membrane).

Statistical analysis

The statistical analysis of the results was performed by Pearson χ^2 test using Statview 4.01 software. Differences were considered statistically significant at values of $P < 0.05$ and suggestively different at values of $P < 0.10$.

Results

Normal mucosa

pS2 immunostaining was observed in every case of normal gastric mucosa. pS2 expression was seen throughout the superficial and foveolar epithelium of antrum, incisura angularis and body (Figure 1), as well as in mucopeptic cells of the neck. pS2 immunostaining was also seen, focally, in antrum glands but not in body glands. The intensity of the staining was much stronger in the superficial part of the mucosa than in the glands. At the cellular level the pattern of pS2 expression was mainly cytoplasmic (diffuse), but strong immunostaining was also seen in the perinuclear region (Golgi area), apical membrane and luminal secretions.

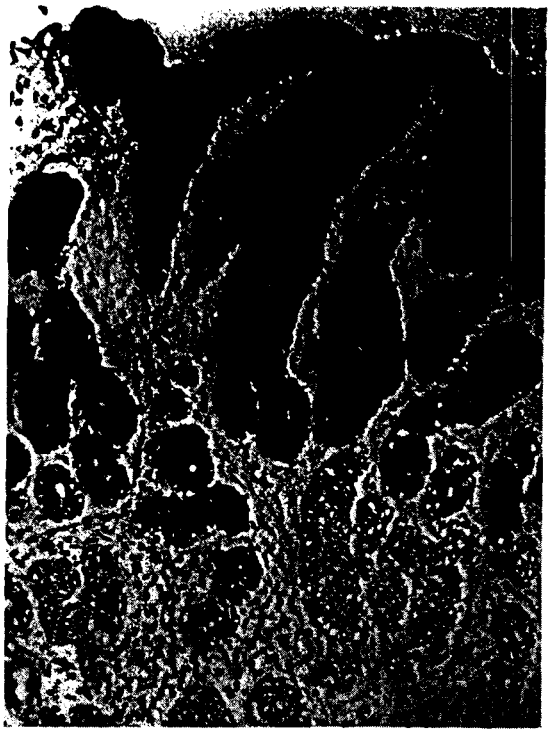

Figure 1. Normal stomach: The superficial and foveolar epithelium of the antral mucosa displays intense pS2 immunoreactivity, × 140.

IM in gastric biopsies from dyspeptic patients

In this group there were six cases of complete (type I) IM and 12 cases of incomplete [types II ($n = 6$) and III ($n = 6$)] IM. pS2 expression was found in every case of IM. There was a clear difference between the two types: in complete IM, pS2 expression was restricted to goblet cells (Figure 2a), whereas in incomplete IM pS2 was expressed in both goblet and columnar cells (Figure 2b). In columnar cells, the intensity of immunoreactivity was weaker than in gastric epithe-

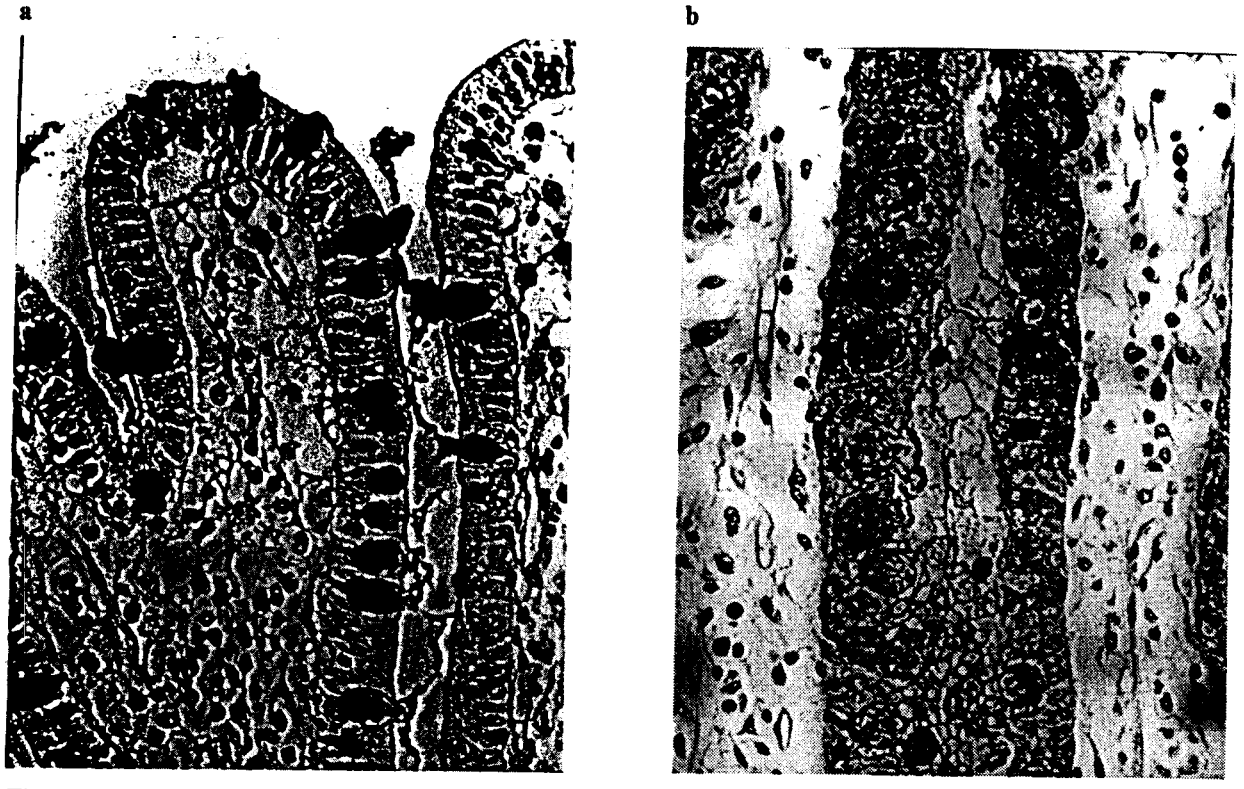

Figure 2. pS2 immunoreactivity in intestinal metaplasia (IM): **a** restricted to goblet cells in complete IM; **b** observed both in goblet and columnar cells of incomplete IM × 700.

Table 1. Relationship between pS2 immunoreactivity and gastric polyp type

Polyp type	Immunoreactivity (% of cells)		
	0	<50	>50
Hyperplastic ($n = 10$)	0 (0)	0 (0)	10 (100)
Adenomatous ($n = 11$)	3 (27.3)	7 (63.6)	1 (9.1)

P value = 0.0002.

lium. No differences were found between types II and III of incomplete IM.

Gastric polyps

In hyperplastic polyps, pS2 was expressed in every case and always in more than 50% of the cells (Figure 3a, Table 1). At the cellular level, pS2 was expressed in the perinuclear region, diffusely in the cytoplasm and at the apical membrane, as well as in secretions.

In adenomatous polyps, pS2 expression was found in eight out of 11 cases (72.7%; Table 1) and the intensity of staining was generally weaker than that of hyperplastic polyps. In seven cases (63.6%) pS2 was expressed focally, in less than 50% of the cells (Table 1), usually in the apical cytoplasm or in single goblet cells. In the remaining positive case (9.1%), pS2 was detected in more than 50% of the cells (Table 1). In the three negative cases (27.3%) the absence of pS2 expression in the adenomatous lesion contrasted with its prominence in the surrounding gastric mucosa (Figure 3b).

The percentage of cases expressing pS2 in the majority of cells was significantly higher ($P = 0.0002$) in hyperplastic polyps than in adenomatous polyps (Table 1).

Histochemical study of the expression of neutral mucins (PAS staining) and acid mucins (HID/AB) was performed in nine hyperplastic polyps (there was no material available from one case) and in the 11 adenomatous polyps. The results, summarized in Table 2, show that hyperplastic polyps secrete mainly neutral mucins, which were expressed, in every case, in more than 50% of the cells. Acid mucins were found in five out of nine hyperplastic polyps (55.6%), always in less than 50% of the cells, with a predominance of sialomucins (Table 2). Neutral mucins were detected in every adenomatous polyp, either focally and mainly in the apical cytoplasm of the cells (seven cases) or in

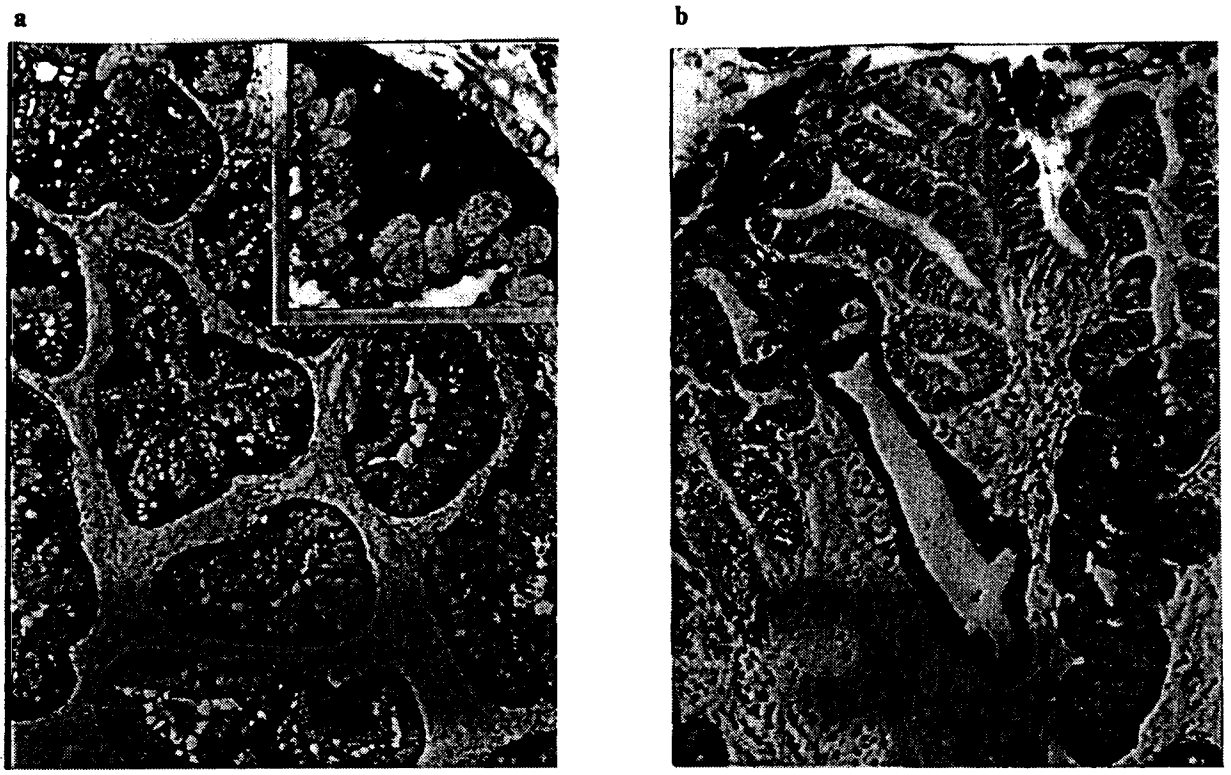

Figure 3. pS2 immunoreactivity in gastric polyps. **a** Hyperplastic polyp: immunoreactivity is observed in almost every cell. *Inset*: higher magnification showing the localization of pS2 immunoreactivity in the perinuclear area and also, although less intensely, in the cytoplasm of the cells. **b** Adenomatous polyp: the glands of the adenomatous lesion do not display pS2 immunoreactivity, in contrast with the intense immunostaining observed in the adjacent gastric mucosa. **a** × 140, Inset × 700; **b** × 350.

more than 50% of the cells (four cases; Table 2). Acid mucins were observed, focally, in every adenomatous polyp (Table 2). In serial sections, it was possible to demonstrate that pS2 positive cells were systematically positive for mucin staining.

Mucosa adjacent to gastric carcinomas

The mucosa adjacent to gastric carcinomas displayed either superficial gastritis (six cases) or chronic atrophic gastritis (44 cases). The pS2 expression in foveolar and surface epithelium of non-neoplastic gastric mucosa adjacent to carcinomas was similar to that observed in normal mucosa. In 23 cases of chronic atrophic gastritis there were foci of IM. In these lesions the pattern of pS2 expression was similar to that observed in IM present in gastric biopsies from dyspeptic patients. In complete (type I) IM pS2 expression was restricted to goblet cells. In incomplete (types II and III) IM pS2 was observed both in goblet and columnar cells.

Gastric carcinomas

pS2 immunostaining was found in 33 out of the 50 gastric carcinomas (66.0%). pS2 immunostaining was observed in 16 out of 18 (88.9%) diffuse carcinomas and in 15 out of 28 (53.6%) intestinal carcinomas, and this difference was found to be statistically significant ($P = 0.037$). Of the diffuse carcinomas, 22.2% showed immunoreactivity in the majority of neoplastic cells (> 50% of the cells; Figure 4). The corresponding frequency in intestinal carcinomas was 10.7% (Figure 5).

No significant correlations were found between pS2 immunostaining and the gender or age of patients, stage of the neoplastic disease, depth of penetration of gastric wall, lymph node metastasization and venous invasion.

The study of several sections, demonstrated a topographic overlap between pS2 expression and mucin secretion, regardless of the histological type of the carcinomas.

Table 2. pS2 and mucin expression in hyperplastic and adenomatous polyps of the stomach

Case no.	Type	Dysplasia	pS2 (% of cells)	Mucin histochemistry		
				Neutral (% of cells)	Acid	
					(% of cells)	Type
1	H	–	>50	>50	–	–
2	H	–	>50	>50	<50	Si+Su
3	H	–	>50	>50	<50	Si
4	H	–	>50	NA	NA	NA
5	H	–	>50	>50	<50	Si
6	H	–	>50	>50	–	–
7	H	–	>50	>50	<50	Si+Su
8	H	–	>50	>50	–	–
9	H	–	>50	>50	<50	Si+Su
10	H	–	>50	>50	–	–
11	Ad	Moderate	–	<50	<50	Si+Su
12	Ad	Moderate	–	<50	<50	Si+Su
13	Ad	Moderate	–	>50	<50	Si+Su
14	Ad	Moderate	<50	<50	<50	Si+Su
15	Ad	Moderate	<50	>50	<50	Su
16	Ad	Severe	<50	<50	<50	Si+Su
17	Ad	Severe	<50	<50	<50	Su
18	Ad	Moderate	<50	>50	<50	Si+Su
19	Ad	Moderate	<50	<50	<50	Su
20	Ad	Moderate	<50	<50	<50	Su
21	Ad	Moderate	>50	>50	<50	Si+Su

H, hyperplastic polyp; Ad, adenomatous polyp; Si, sialomucins; Su, sulphomucins; NA, not available for histochemical study.

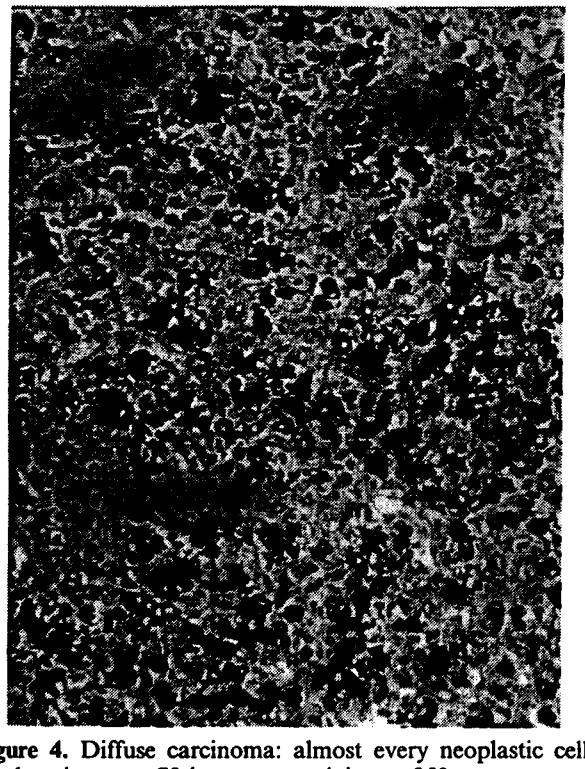

Figure 4. Diffuse carcinoma: almost every neoplastic cell displays intense pS2 immunoreactivity. × 350.

Discussion

Trefoil peptides are members of a family of small secreted molecules, with 1–6 highly conserved 6-cysteine-rich regions (P-domains) which are responsible for their distinctive three-loop structure (Thim *et al*, 1985; Hauser and Hoffmann, 1992).

These peptides have been found in many species from amphibians to mammals and have been shown to be closely associated with mucus-secreting epithelia (Wright *et al*, 1993). In humans, three trefoil peptides have been identified to date (pS2/BCEI, hSP/SML1 and hITF/ITF1). Their distribution in the gastrointestinal mucosa follows a specific pattern: for example, in the gastric mucosa pS2 and hSP are co-expressed in foveolar and superficial epithelium, whereas hSP alone is seen in pyloric and Brunner glands (Wright *et al*, 1990; Hanby *et al*, 1993b) and hITF is mainly expressed in small intestinal and colonic goblet cells (Hauser *et al*, 1993; Podolsky *et al*, 1993). In ulcerative conditions of the gastrointestinal tract it has been shown that trefoil peptides, mainly pS2 and hSP, are expressed at the periphery of mucosal ulcerative lesions in the so-called ulcer-associated cell lineage (Wright *et al*, 1990, 1993; Rio *et al*, 1991). Trefoil peptides have also been detected

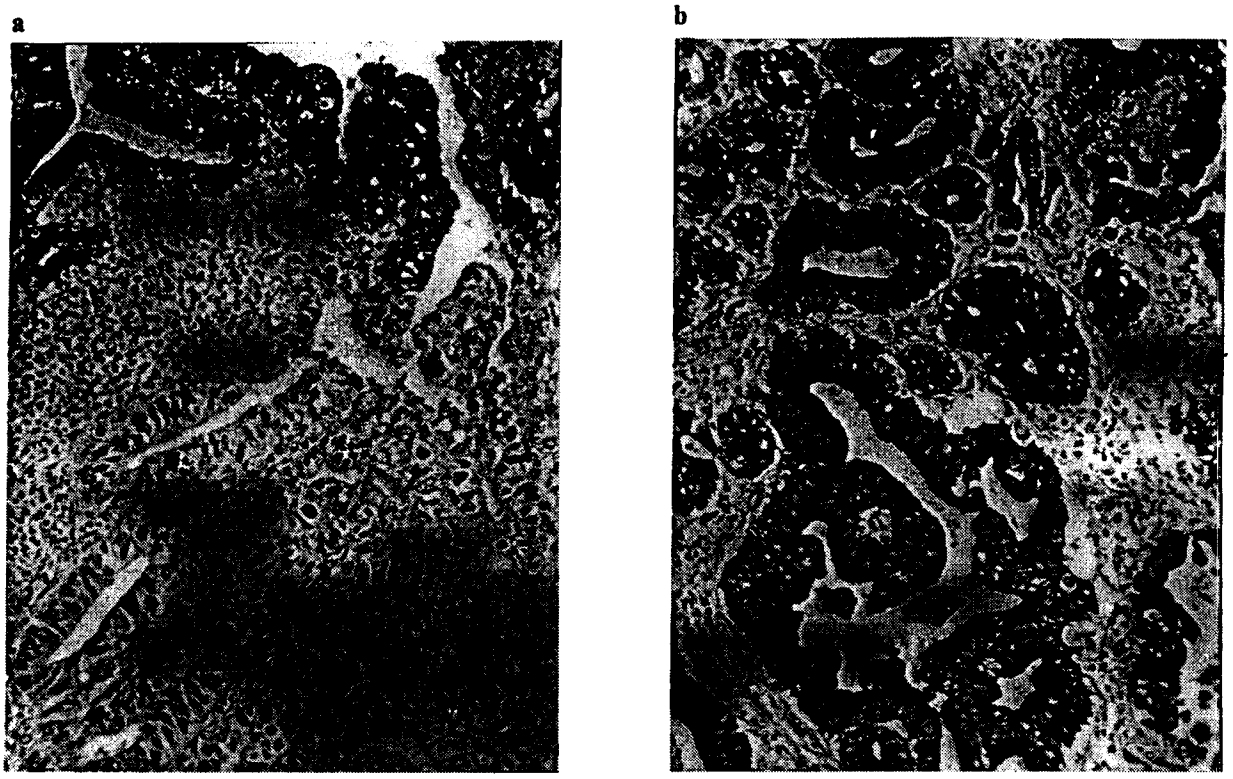

Figure 5. pS2 immunoreactivity in intestinal carcinomas: **a** pS2-negative intestinal carcinoma. Note the intense pS2 immunoreactivity in the adjacent gastric epithelium. **b** pS2-positive intestinal carcinoma. Most of the neoplastic cells display intense pS2 immunoreactivity × 350.

in other pathological conditions of the gastrointestinal tract, such as Barrett's oesophagus (Hanby *et al*, 1994) and gastric metaplasia of proximal duodenum (Hanby *et al*, 1993a).

pS2 is the trefoil peptide that has been most extensively studied in humans, both in normal and neoplastic tissues (for a thorough review see Thim, 1994). In human breast cancer pS2 expression was originally reported as being restricted to a sub-group of tumours expressing oestrogen receptors (Rio *et al*, 1987) and associated with hormone therapy responsiveness (Henry *et al*, 1990). In the stomach, pS2 expression is oestrogen-independent since oestrogen receptors have not been found in this organ in either non-neoplastic mucosa or carcinomas (Rio *et al*, 1988; Machado *et al*, 1994).

In the present study we analysed the pattern of pS2 immunohistochemical expression in normal gastric mucosa and premalignant and malignant lesions of the stomach. To the best of our knowledge this is the first study to provide a thorough description of pS2 expression in premalignant lesions of the stomach such as chronic atrophic gastritis, IM and gastric polyps.

Our findings in normal gastric mucosa confirm those previously reported by other authors (Rio *et al*, 1988; Luqmani *et al*, 1989; Hanby *et al*, 1993b; Müller and Borchard, 1993). pS2 expression was detected in the foveolar and superficial epithelium of gastric mucosa, both in the antrum and body/fundus. Immunostaining was observed in every case, at slight variance with the results reported by Müller and Borchard (1993) who found pS2 immunostaining in 95% of the cases included in their series. At variance with other studies (Rio *et al*, 1988; Hanby *et al*, 1993b), we observed also focal and weak immunostaining for pS2 in mucus glands of the gastric antrum. The pS2 expression in gastric epithelium in chronic gastritis was similar to that observed in normal mucosa.

In every instance (foveolar/superficial epithelium, IM, polyps and carcinomas) we found a good correlation between the amount and topographic distribution of pS2 positive cells and mucus secreting

cells, in keeping with the consistent co-expression of pS2 and mucins advanced by Wright *et al* (1993).

We observed pS2 immunostaining in every case of IM, in agreement with Hanby *et al* (1993b) and at variance with the findings of Luqmani *et al* (1989) and Müller and Borchard (1993). We observed, furthermore, that the features of pS2 expression in metaplastic lesions are IM type-specific and independent of the presence of gastric carcinoma in the neighbourhood. Our results demonstrate distinctive patterns of pS2 expression in complete and incomplete IM. In the former pS2 expression is restricted to goblet cells and in the latter the staining is observed both in goblet and columnar cells. The pattern of pS2 expression is similar in the two types (II and III) of incomplete IM. These findings strongly support the aforementioned co-expression of pS2 and mucins, since columnar cells in incomplete IM are mucus-producing cells, whereas in complete IM, columnar cells are non-mucus-producing, mature absorptive cells. Since Hanby *et al* (1993b) found pS2 expression only in the goblet cells of their series of IM it is tempting to suggest the cases studied by Hanby *et al* (1993b) probably displayed solely the complete type of IM.

Our results regarding pS2 expression in the different types of IM raise two additional issues. The first concerns type III IM, which is designated by some authors, colonic metaplasia (Sipponen *et al*, 1980; Correa, 1992). Our finding of pS2 expression in goblet and columnar cells in this type of IM contrasts with the pattern of pS2 expression in the colon, where it has been reported either as absent (Rio *et al*, 1988; Piggott *et al*, 1991; Hanby *et al*, 1993c) or present in only a few goblet cells (Welter *et al*, 1994). Our finding thus reinforces the contention of Filipe and Jass (1986) as well as our own (Carneiro *et al*, 1994) that use of the term 'colonic metaplasia' to designate this type of IM is inappropriate and misleading.

The second issue concerns the sequential evolution of complete to incomplete IM types, along the process of malignant transformation of gastric mucosa, as suggested, based mainly upon epidemiologic evidence, in Correa's model of gastric carcinogenesis (Correa *et al*, 1990a,b; Correa, 1992). Although realizing the limitations of our study in addressing such a problem we think we have obtained enough data to claim that, in contrast to complete IM, the metaplasia of incomplete IM 'still' displays features of gastric differentiation, as shown by pS2 immunostaining in columnar mucus-secreting cells. We feel thus tempted to advance that the complete and incomplete types of IM may represent two alternative pathways of metaplastic change in gastric mucosa, rather than two successive steps of phenotypic changes, with complete IM leading to incomplete IM, as suggested in Correa's model. It remains unclarified, however, whether the different IM types represent peculiar responses to different environmental factors or specific adaptations related to physiopathological conditions of the gastric epithelium.

We observed a clear difference between hyperplastic and adenomatous polyps. The former display a pattern of pS2 expression which is similar to that observed in normal foveolar and surface epithelium. In hyperplastic polyps we observed, moreover, a good correlation between pS2 expression and the production of mucins, mainly the neutral mucins which are the mucins normally expressed in gastric mucosa. These results fit with the assumption that hyperplastic polyps represent proliferative epithelial lesions which are phenotypically similar to normal gastric epithelium, but they do not provide conclusive evidence for or against our previous contention that foveolar hyperplasia/hyperplastic polyps may serve as precursor lesions of gastric carcinoma of the diffuse type (Carneiro *et al*, 1993a,b, 1995a).

At variance with hyperplastic polyps, pS2 was detected only in 72.7% of the adenomatous polyps and, in the positive cases, the percentage of immunoreactive cells was significantly lower than in hyperplastic polyps. This down-regulation of pS2 expression in adenomatous lesions supports the idea that adenomatous polyps are precursor lesions of gastric carcinoma of intestinal type, in which we also observed a reduced expression of pS2.

In gastric carcinomas we found pS2 expression in 66.0% of the cases, a frequency that is close to those reported by Luqmani *et al* (1989), Henry *et al* (1991) and Müller and Borchard (1993) (57, 56 and 48%, respectively) and substantially inferior to that reported by Theisinger *et al* (1991). Differences in the methodologies and criteria used in the five series probably account for the aforementioned discrepancy. We observed a significantly higher frequency of pS2 immunoreactivity in diffuse carcinomas (88.9%) than in intestinal carcinomas (53.6%). Furthermore, we observed that the frequency of carcinomas with a high percentage of immunoreactive cells (> 50%) was higher in diffuse than in intestinal carcinomas (22.2 and 10.7%, respectively). These findings concur with those of Theisinger *et al* (1991) and contrast with the results obtained by Müller and Borchard (1993) who did not find any significant relationship between pS2 expression and the histologic type of gastric carcinoma.

Müller and Borchard (1993) found a highly significant correlation between pS2 immunoreactivity and expression of markers of gastric differentiation, such as pepsinogen II and 2B5, which were co-expressed in most of the pS2 immunoreactive cells. If we assume, as Müller and Borchard (1993) did, that pS2 immunoreactivity discloses the gastric phenotype of neoplastic cells, we may conclude that most diffuse carcinomas (88.9%) display gastric-type differentiation, which is exhibited in the majority of the neoplastic cells in about one-fifth of the cases. These findings are in accordance with ultrastructural data, showing that gastric-type (foveolar and/or mucopeptic) cells are observed in the majority of diffuse carcinomas (Fiocca *et al*, 1987; Carneiro *et al*, 1992). Furthermore, about half of the so-called intestinal carcinomas of our series—those displaying pS2 immunostaining—exhibited focal (42.9% of the cases) or extensive (10.7% of the cases) signs of gastric-type differentiation. These findings concur with those of Fiocca *et al* (1988), in whose series 55% of the cases of gastric carcinoma with glandular structure expressed pepsinogen II, and with those of Kushima and Hattori (1993), who showed that gastric-type differentiation was present exclusively (23.2%) or in association with intestinal-type differentiation (46.5%) in a series of 43 early gastric carcinomas with glandular structure.

The present and the aforementioned studies show that gastric-type differentiation is present in both diffuse carcinomas and intestinal carcinomas, although much more often and more expressively in the former than in the latter. The prominence of gastric-type differentiation in diffuse carcinomas suggests that this is the type of carcinoma which is more closely linked to the gastric mucosa both histogenetically and from the differentiation standpoint, whereas the group of intestinal carcinomas encompasses gastric-type adenocarcinomas, carcinomas with intestinal-type differentiation and tumours with dual differentiation. The heterogeneity of this latter group reinforces our previous contention that gastric carcinomas with gland formation should be designated as 'glandular' carcinomas instead of 'intestinal' carcinomas (Carneiro *et al*, 1995b), in order to avoid the mixture of structural and cell differentiation concepts.

The existence of glandular adenocarcinomas with gastric-type differentiation challenges the universality of Correa's model regarding the histogenesis of the intestinal carcinomas of Laurén's classification. Without denying the intestinal pathway of gastric carcinogenesis, whose magnitude still remains unclarified, one has to admit also the possibility of development of gastric carcinoma with glandular structure from gastric proper mucosa, either *de novo* or via hyperplastic lesions, as suggested by several authors (Hattori, 1985; Watanabe *et al*, 1992; Kushima and Hattori, 1993). Our finding of a similar pattern of pS2 immunoexpression in foveolar/surface epithelium, hyperplastic lesions and some glandular carcinomas with gastric-type differentiation is in keeping with the latter possibility.

Taking all the above evidence together, we think one should admit the existence of at least two pathways of malignant transformation of gastric mucosa: one via intestinal metaplasia and adenomatous (flat or polypoid) dysplasia, leading to glandular carcinomas with intestinal-type differentiation, and the other via hyperplastic or *de novo* changes, with or without concurrent non-metaplastic dysplasia, leading to isolated-cell carcinomas (diffuse carcinomas of Laurén's classification) and to a subset of glandular carcinomas with gastric-type differentiation. The carcinomas with dual differentiation raise the possibility of a phenotypic shift from gastric- to intestinal-type differentiation during the progression of some gastric-type adenocarcinomas.

Thus, our results show that there are major changes in the immunoexpression of pS2 protein along the process of gastric carcinogenesis, which are reflected in the specific patterns of pS2 immunoreactivity observed in the different types of IM and gastric polyps. In gastric carcinomas, pS2 expression can be used as a marker of gastric-type differentiation and is associated with gastric carcinomas of the diffuse type and with a subset of glandular carcinomas.

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PAPER II

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Original Paper

pS2 Protein Expression in Gastric Carcinoma. An Immunohistochemical and Immunoradiometric Study

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The aim of this study was to examine the prevalence of pS2 expression in gastric carcinoma and to determine its prognostic significance. We analysed pS2 protein expression in 50 gastric carcinomas and respective adjacent mucosas by immunohistochemistry and immunoradiometric assay (IRMA). pS2 was consistently expressed in superficial and foveolar epithelium of non-neoplastic mucosa and in 66.0% of the carcinomas. pS2 immunoreactivity was significantly higher in diffuse than in intestinal carcinomas, and in those cases with nodal metastases than in those without. No correlation was found between pS2 immunostaining and gender, age, staging, depth of wall penetration, venous invasion, ploidy and S-phase fraction. The mean levels of pS2 (IRMA) were significantly lower in gastric carcinomas than in non-neoplastic mucosas, and were not correlated with any of the aforementioned clinicopathological features. The survival of patients with pS2-positive tumours was not significantly different from that of patients with pS2-negative tumours. We conclude that pS2 expression, which can be used as a marker of gastric-type differentiation, is associated with gastric carcinoma of diffuse type. The lack of correlation between pS2 expression and most features of tumour aggressiveness and patients' survival precludes its use as a prognostic tool in gastric carcinoma.

Key words: gastric carcinoma, immunohistochemistry, immunoradiometric assay (IRMA), mucins, pS2, stomach, survival, trefoil peptides

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INTRODUCTION

pS2 PROTEIN is a member of the trefoil peptide family whose gene was cloned from the MCF-7 breast cancer cell line by Masiakowski and colleagues [1]. In human breast cancer, pS2 is associated, though not exclusively, with oestrogen receptor expression [2–4], responsiveness to hormone therapy [5] and favourable prognosis [6, 7].

In normal human tissues, and excluding the pS2 expression in some apparently normal breast specimens [4, 8], pS2 has only been consistently detected in the superficial and foveolar epithelium of gastric mucosa [9–14].

The few published reports on pS2 immunoexpression in gastric carcinoma [10, 14–16] provide discrepant data on some of the clinicopathological parameters under analysis. Theisinger and colleagues [16] observed pS2 expression of variable intensity in every carcinoma of their series, whereas the prevalence of pS2 immunoreactivity varied from 48 to 57 per cent in other series [10, 14, 15]. In contrast to Müller and Borchard [14] who did not find any significant relationship between pS2 expression and the histological type, Theisinger and colleagues [16] found a close association between diffuse gastric carcinoma and a high percentage of strongly stained pS2 immunoreactive cells. Finally, Müller and Borchard [14] reported a significant relationship between pS2 expression and extent of tumour growth (pT stage); despite this, they did not observe any significant influence of the immunohistochemical expression of pS2 on the outcome of the patients [14].

We undertook the present immunohistochemical and immunoradiometric study of pS2 in a series of gastric carcinomas in an attempt to settle the aforementioned discrepancies. We also intended to determine whether the immunohistochemical evaluation of pS2 carries any meaningful

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prognostic information as has been observed in mammary and pulmonary adenocarcinomas [6, 17].

MATERIALS AND METHODS

Tissue material

Six cases of normal gastric biopsies obtained from dyspeptic patients without gastric carcinoma or other focal lesions at endoscopy were included in this study. From each patient, there were six biopsies available (two from the antrum, two from the incisura and two from the body/fundus). Surgical specimens from 50 gastric carcinomas consecutively resected at Hospital S. João-Medical Faculty (IPATIMUP), Porto, Portugal, were studied. The mucosa adjacent to each case of gastric carcinoma was also studied.

The tissue fragments were fixed in 10% formaldehyde and embedded in paraffin. Serial sections of 4 μm were cut from each block and used for routine staining with haematoxylin and eosin (H&E), periodic acid Schiff (PAS) and alcian blue pH 2.5/high iron diamine (HID/AB) and immunohistochemical stains. Frozen samples from 33 carcinomas were available for flow cytometry performed as previously described [18].

Immunohistochemistry

Monoclonal antibody (MAb) BC4 (CIS bio international, Gif-Sur-Yvette, France) was used for immunohistochemical (IHC) study of the expression of pS2 in formalin-fixed paraffin-embedded tissues. A modification of the avidin-biotin-peroxidase complex method [19] was applied. The paraffin sections (4 μm thick) were dewaxed, incubated for 30 min at 37°C in a 0.01 M HCl solution containing 0.4% pepsin and then rinsed in TRIS buffered saline (TBS), pH 7.6. The sections were treated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase, washed in TBS, and then incubated for 20 min with normal rabbit serum, at a dilution of 1:5 in TBS containing 25% bovine serum albumin (BSA). Excess normal serum was removed and replaced by the MAb BC4 diluted 1:4. After overnight incubation (18 h) at 4°C, slides were washed with TBS and the sections incubated with a 1:200 dilution of biotin-labelled secondary antibody for 30 min. After washing with TBS, sections were incubated with avidin-biotin-peroxidase complex (10 mg/ml of biotin-labelled peroxidase) for 60 min. This was followed by staining the sections for 5 min with 0.05% diaminobenzidine, freshly prepared in 0.05 M TRIS buffer, pH 7.6, containing 0.01% hydrogen peroxide. Finally, sections were counterstained with haematoxylin, dehydrated and mounted. Dilution of primary antibodies, biotin-labelled secondary antibodies, and avidin-biotin-peroxidase complex were made with TBS containing 12.5% BSA.

All series included normal gastric mucosa as positive controls. Negative controls were performed by substitution of the primary antibody with IgG1 immunoglobulins of the same subclass and concentration as the MAb.

Scoring

A case was considered to be negative (0) whenever histochemical or immunohistochemical staining was absent or present in only very few cells. A semiquantitative approach was used to score the staining of the positive cases into cases with moderate number of positive cells (+) and cases in which the majority of the neoplastic cells were immunoreactive (++), irrespective of the localisation of the positive cells and the intensity of the staining.

Immunoradiometric assay (IRMA) for pS2

Immunoradiometric assay for pS2 was performed in 30 tumours and 12 samples of non-neoplastic mucosa. From every case, a parallel sample of neoplastic or non-neoplastic tissue was obtained in order to monitor, by histological examination, if the IRMA samples were representative. Frozen tissues were homogenised at 0°C in a Polytron homogeniser. Homogenization buffer, pH 7.4, included 10 mM Tris, 1.5 nM EDTA, 1 mM ditiothreitol, 1% v/v monotioglycerol, and 10% v/v glycerol. The homogenates were centrifuged at 105000 *g* at 0°C, for 70 min. The obtained supernatant fraction was used for immunoradiometric assay and for total protein quantification according to Bradford [20]. pS2 immunoradiometric assay kit ELSA-pS2 was purchased from CIS bio international (Gif-Sur-Yvette, France) and used according to the supplier's recommendations. Briefly, samples were incubated with pS2 ^{125}I-radiolabelled monoclonal antibody in pS2 monoclonal antibody coated tubes (standards and tissue supernatant fractions) at room temperature for 1 h. Unbound radiolabelled antibody was removed by washing the tubes and radioactivity was measured.

Statistical analysis

The results are expressed as a percentage or as a mean ± standard deviation. The statistical analysis of the results was performed by Pearson-chi squared test, unpaired Student's *t*-test and Mann–Whitney *U* test using Statview 4.01 software. Follow-up data was obtained in every case. The median follow-up was 17 months (range 3–63 months). Survival curves were calculated according to the Kaplan–Meier method and statistically compared using the Mantel–Cox test using BMDP statistical software package. Differences were considered to be statistically significant at values of $P<0.05$.

RESULTS

Normal mucosa

We found pS2 immunostaining in every case of normal gastric mucosa. pS2 expression was seen throughout the superficial and foveolar epithelium of antrum and body (Figure 1), as well as in mucopeptic cells of the neck. pS2 immunostaining was also seen, focally, in antrum glands but not in body glands. In the antral glands, the intensity of immunostaining was much weaker than in the superficial part of the mucosa. At the cellular level, the pattern of pS2 expression was mainly cytoplasmic (diffuse) but strong immunostaining was also seen in the perinuclear region (Golgi area), apical membrane and luminal secretions (Figure 1).

Mucosa adjacent to gastric carcinomas

The mucosa adjacent to gastric carcinomas displayed either superficial gastritis (six cases) or chronic atrophic gastritis (44 cases). The pS2 expression in foveolar and surface epithelium of non-neoplastic gastric mucosa adjacent to carcinomas was similar to that of normal mucosa.

Gastric carcinoma

Table 1 summarises the clinicopathological features of the 50 cases included in the present series and the immunohistochemical findings regarding pS2 expression. All 50 carcinomas were classified according to Laurén [21] as intestinal ($n = 28$), diffuse ($n = 18$) and unclassifiable ($n = 4$). pS2 immunostaining was observed in 33 of the 50 gastric carcinomas (66.0%),

Figure 1. Normal gastric mucosa: pS2 immunoreactivity is observed throughout the superficial and foveolar epithelium of antral mucosa (original magnification × 140).

which included 16 of 18 (88.9%) diffuse carcinomas (Figure 2) and 15 of 28 (53.6%) intestinal carcinomas (Figure 3). This difference was found to be statistically significant ($P = 0.037$) (Table 1). The frequency of cases exhibiting immunoreactivity in the majority of neoplastic cells (++) was higher in diffuse (22.2%) than in intestinal (10.7%) carcinomas.

Sixteen cases, classified according to the predominant histological type as intestinal or diffuse carcinomas, displayed small

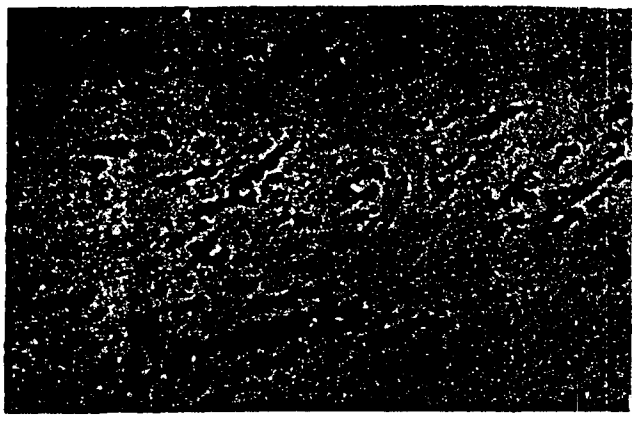

Figure 2. pS2-positive diffuse carcinoma: intense pS2 immunoreactivity is observed in almost every neoplastic cell (original magnification × 350).

Table 1. Relationship between the clinicopathological features of gastric carcinomas and pS2 expression

		pS2 IHC		
	Number of cases (%)	Negative	Positive	*P* value
Gender				
Male	34 (68.0)	12 (35.3)	22 (64.7)	
Female	16 (32.0)	5 (31.2)	11 (68.8)	NS
Age				
<40 years	9 (18.0)	2 (22.2)	7 (77.8)	
≥40 years	41 (82.0)	15 (36.6)	26 (63.4)	NS
Stage				
I	23 (46.0)	9 (39.1)	14 (60.9)	
II	10 (20.0)	6 (60.0)	4 (40.0)	
III	16 (32.0)	2 (12.5)	14 (87.5)	
IV	1 (2.0)	0 (0)	1 (100)	NS
Histological type				
Intestinal	28 (56.0)	13 (46.4)	15 (53.6)	
Diffuse	18 (36.0)	2 (11.1)	16 (88.9)	
Unclassifiable	4 (8.0)	2 (50.0)	2 (50.0)	0.037
Depth of invasion				
Mucosa and submucosa	9 (18.0)	2 (22.2)	7 (77.8)	
Muscular and serosa	41 (82.0)	15 (36.6)	26 (63.4)	NS
Metastases to the lymph nodes				
Negative	28 (56.0)	13 (46.4)	15 (53.6)	
Positive	22 (44.0)	4 (18.2)	18 (81.8)	0.036
Venous invasion				
Negative	29 (58.0)	11 (37.9)	18 (62.1)	
Positive	21 (42.0)	6 (28.6)	15 (71.4)	NS
Ploidy ($n = 33$)				
Diploid	16 (48.5)	8 (50.0)	8 (50.0)	
Aneuploid	17 (51.5)	7 (41.2)	10 (58.8)	NS
Total	50 (100)	17 (34)	33 (66)	

NS, not significant.

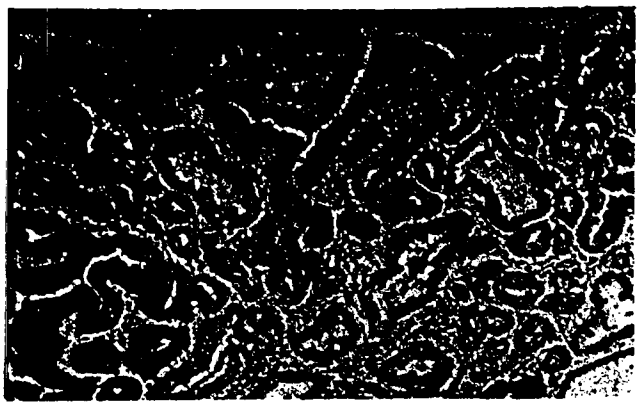

Figure 3. pS2-positive intestinal carcinoma: pS2 immunoreactivity is observed in less than 50% of the neoplastic cells (original magnification × 140).

foci with a structure different from the main component of the tumour—foci of intestinal carcinoma in diffuse carcinomas ($n = 10$) and foci of diffuse carcinomas in intestinal carcinomas ($n = 6$). pS2 immunoreactivity was observed in every one of these 'mixed' carcinomas. In most cases ($n = 12$), immunoreactivity was observed in both the diffuse and the intestinal component of the tumours (Figure 4). In the remaining four cases, immunostaining was restricted to the diffuse component (Figure 5).

In the whole series we found a significant correlation between pS2 immunostaining and metastasis to lymph nodes ($P = 0.036$) (Table 1). Within each group of the different histological types, no significant correlation was found between pS2 expression and lymph node metastases (data not shown).

No significant correlations were found between pS2 immunostaining and the gender or age of patients, stage of the neoplastic disease, depth of penetration of gastric wall and venous invasion (Table 1). Similarly, no significant correlation was observed with regard to ploidy (Table 1) and S-phase fraction (SPF) (16.8 ± 16.5% in the positive cases and 17.4 ± 10.3% in the negative cases).

The comparison of the pS2 immunoexpression with mucin histochemical expression in serial sections of the gastric carcinomas revealed that there was a close topographic overlap

Figure 4. In this 'mixed' carcinoma pS2 immunoreactivity is observed in both components of the tumour: intestinal component (left) and diffuse component (right) (original magnification × 350).

Figure 5. In this 'mixed' carcinoma pS2 immunoreactivity is restricted to one of the components of the tumour: diffuse component (left) with positive cells and intestinal component (right) with no immunoreactive cells (original magnification × 350).

between both stainings, regardless of mucin type (neutral and/or acid) and the histological type of the tumours.

The mean level of pS2 in gastric carcinomas as measured by IRMA was significantly lower ($P = 0.05$) than that of non-neoplastic mucosa (58.8 ± 42.6 and 76.5 ± 21.6, respectively). pS2 mean level detected by IRMA was higher in immunohistochemical positive cases (64.5 ± 46.9) than in negative cases (52.3 ± 37.8), but the difference did not attain the threshold of statistical significance. No significant correlation was found between the mean levels of pS2 and the different clinicopathological features of the cases, namely the histological type of the carcinomas and the presence of nodal metastases.

The comparison of the postoperative survival curves of patients with pS2-positive and patients with pS2-negative gastric carcinomas revealed no significant difference between the two groups (Figure 6).

DISCUSSION

We found pS2 expression in 66.0% of the gastric carcinomas, a frequency that is close to those reported by Luqmani and colleagues [10], Henry and colleagues [15] and Müller and Borchard [14] (57, 56 and 48%, respectively) and substantially inferior to that reported by Theisinger and colleagues [16]. Differences in the methodologies and criteria used in the five series probably account for the discrepancies.

We observed a significantly higher frequency of pS2 im-

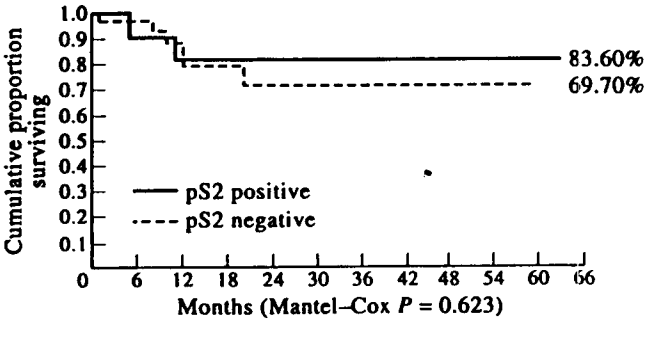

Figure 6. Survival curves of patients with pS2-positive and pS2-negative gastric carcinoma.

munoreactivity in diffuse carcinomas (88.9%) than in intestinal carcinomas (53.6%). Furthermore, we observed that the frequency of carcinomas displaying immunoreactivity in the majority of neoplastic cells was higher in diffuse than in intestinal carcinomas (22.2 and 10.7%, respectively). These findings concur with those of Theisinger and colleagues [16] and contrast with the results obtained by Müller and Borchard [14] who did not find any significant relationship between pS2 expression and the histological type of gastric carcinoma. The close relationship we found between pS2 expression and carcinomas of the diffuse type was also observed in some of the 'mixed' carcinomas of the present series, which displayed immunoreactivity in the diffuse component in contrast to the absence of immunostaining in the intestinal component.

Müller and Borchard [14] found a highly significant correlation between pS2 immunoreactivity and expression of markers of gastric differentiation, such as pepsinogen II and 2B5, which were co-expressed in most of the pS2 immunoreactive cells. If we assume, as Müller and Borchard [14] did, that pS2 immunoreactivity discloses the gastric phenotype of neoplastic cells, we may conclude that most diffuse carcinomas (88.9%) display a gastric-type differentiation, which is exhibited in the majority of the neoplastic cells in about one-fifth of the cases. These findings are in accordance with the evidence provided by ultrastructural studies, which have shown that gastric-type cells (foveolar and/or mucopeptic) are observed in the majority of diffuse carcinomas [22, 23]. Furthermore, almost half of the intestinal carcinomas of our series—those displaying pS2 immunostaining—exhibited focally (42.9% of the cases) or extensively (10.7% of the cases) signs of gastric-type differentiation. These findings are in agreement with the data of Fiocca and colleagues [24] who showed that, in their series, 55% of the cases of gastric carcinoma with glandular structure expressed pepsinogen II. Kushima and Hattori [25] searched for signs of gastric and intestinal differentiation using histochemical methods and showed that gastric-type differentiation was present—exclusively or in association with intestinal-type differentiation—in 69.8% of differentiated-type carcinomas (with glandular structure). The evidence provided by ultrastructural studies [22, 23] also demonstrates the presence of different cell types, including foveolar and mucopeptic cells (gastric-type cells), besides intestinal columnar or goblet cells (intestinal-type cells) in gastric carcinomas forming glands [22, 23].

Overall, the present and the aforementioned studies provide enough evidence to claim that gastric-type differentiation is present both in diffuse and intestinal types of gastric carcinoma, though much more often and more expressively in the former than in the latter. The prominence of cellular differentiation of gastric-type in diffuse carcinomas suggests that this is the type of carcinoma which is more closely linked to the gastric mucosa both histogenetically and from a differentiation standpoint, whereas the so-called intestinal carcinomas encompass gastric-type adenocarcinomas, carcinomas with intestinal-type differentiation and tumours with dual differentiation. The latter finding reinforces our previous contention that gastric carcinomas with gland formation should be designated as 'glandular' carcinomas instead of 'intestinal' carcinomas [26], in order to avoid the mixture of structural and cell differentiation concepts.

We found a significant correlation between pS2 expression and lymph node metastases. This finding is a side-effect related to the histological type since, in our series, most of the cases with lymph node metastases were diffuse carcinomas (68.2%) which were also those expressing more often pS2 immunoreactivity. Within each histological type, no significant correlation was found between pS2 immunoreactivity and nodal metastases. We found no other significant correlation of pS2 immunoreactivity and the clinicopathological parameters under analysis. These results contrast with those of Müller and Borchard [14] who found, in a series of 120 gastric carcinomas, a significant relationship between pS2 expression and extent of tumour growth (pT stage).

There was a lack of agreement between the results of immunohistochemical study of pS2 expression and those obtained by IRMA, except for the demonstration of higher levels of pS2 in the non-neoplastic mucosas compared with carcinomas. By IRMA, we did not find any significant correlation between pS2 levels and the different clinicopathological parameters, namely regarding the histological type of the tumours. The discrepancy between immunohistochemistry and IRMA may reflect both the effect of tumoural heterogeneity regarding pS2 expression and the effect of stromal contamination. The latter possibility provides a putative explanation for the finding of similar pS2 levels, detected by IRMA, in diffuse and intestinal carcinomas, in contrast to immunohistochemical results which showed that pS2 expression was significantly higher in diffuse than in intestinal carcinomas, since the abundance of stromal, non-neoplastic tissue, is higher in the former than in the latter. It remains to be seen if the correction of cytosolic values for percentage of epithelial cells in the tumour samples will provide less discrepant results with the two methods, as recently suggested by Willemse and colleagues [27].

At variance with breast cancer, pS2 expression does not correlate in gastric carcinoma with the expression of oestrogen receptors (ER). In 39 of 50 cases included in the present series, we found no expression of female sex hormone receptors (ER and progesterone receptors) in the normal mucosa of the stomach nor in the cells of the carcinomas [28].

In gastric carcinomas, we found a good correlation between the amount and topographic distribution of pS2 positive and mucus-secreting cells. Our preliminary studies (data not shown) on the immunoexpression of MUC-5, in serial sections, indicate a topographic overlap between pS2 positive and MUC-5 positive cells. These findings support the concept that there is a co-expression of pS2 and mucins, as previously suggested by Wright and colleagues [29].

We found no statistically significant difference in the survival of patients with pS2-positive and pS2-negative tumours. Despite the too short follow-up of most cases, our results are in keeping with those obtained by Müller and Borchard [14] in their series of 120 gastric carcinomas, and contrast with the results observed in breast cancer [6] and lung cancer [17], in which pS2 immunoreactivity is associated with a more favourable or a less favourable prognosis, respectively.

In conclusion, our results show that in gastric carcinomas, pS2 expression reflects gastric-type differentiation and is significantly associated to the diffuse type of gastric carcinoma. pS2 expression is neither associated with features of tumour aggressiveness nor influences the survival of patients with gastric carcinoma, thus being of no value for prognostic purposes.

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PAPER III

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PATTERNS OF EXPRESSION OF TREFOIL PEPTIDES AND MUCINS IN GASTRIC POLYPS WITH AND WITHOUT MALIGNANT TRANSFORMATION

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SUMMARY

The expression of two trefoil peptides (TFF1 and TFF2) and four mucins (MUC1, MUC2, MUC5AC, and MUC6) was evaluated by immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR) in 29 gastric polyps, 10 hyperplastic and 19 adenomatous, eight of which displayed malignant transformation. The aims of this study were to characterize the expression profile of these molecules in each type of polyp and to investigate possible modifications of the profile during the process of malignant transformation. All hyperplastic polyps displayed immunoreactivity for TFF1, MUC5AC, and MUC1 in more than 75 per cent of the cells. In adenomatous polyps, three main phenotypes could be identified: complete gastric phenotype (co-expression of TFF1 and MUC5AC)—nine cases (47·4 per cent); incomplete gastric phenotype (TFF1-positive and MUC5AC-negative)—seven cases (36·8 per cent); non-gastric (intestinal) phenotype (no expression of TFF1 or MUC5AC)—three cases (15·8 per cent). Data yielded by immunohistochemistry and RT-PCR showed a good correlation for both TFF1 and TFF2. One hyperplastic and seven adenomatous polyps with villous architecture displayed foci of diffuse and intestinal-type carcinoma, respectively; in all of these cases, MUC1 expression and signs of gastric differentiation were observed in both the non-malignant and the carcinomatous component. It is concluded that gastric differentiation is a feature of hyperplastic polyps and of a subset of adenomatous polyps which is shared by early carcinomas arising in some of these polyps, regardless of the histological type of polyp and of carcinoma. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS—**adenomatous polyps; hyperplastic polyps; gastric carcinoma; mucins; stomach; trefoil peptides**

INTRODUCTION

There are two main types of gastric epithelial polyp: hyperplastic and adenomatous polyps. Hyperplastic polyps are the most common, consisting of an abnormal overgrowth of normal gastric foveolar elements. Adenomatous polyps (adenomas) consist of tubular, villous or tubulo-villous structures covered by dysplastic epithelium.[1,2] The incidence of malignant transformation appears to be much lower in hyperplastic polyps than in adenomatous polyps (2–6 per cent and up to 75 per cent, respectively).[1–6] Based on the presence of signs of intestinal differentiation on the adenomatous polyps and of intestinal metaplasia (IM) in surrounding gastric mucosa, it has been proposed that such polyps may be an intermediate step in the process of malignant transformation leading to intestinal-type gastric carcinoma.[7–10] However, it was recently reported that adenomatous polyps of the stomach can also display gastric-type differentiation and/or a mixed gastric and intestinal phenotype.[7,11]

Trefoil peptides (TFF peptides) are small secreted molecules with 1–6 highly conserved 6-cysteine-rich regions (P-domains) that have been shown to be closely related to mucus-secreting epithelia.[12–15] In humans, three trefoil peptides have been identified to date: TFF1 (pS2), TFF2 (hSP), and TFF3 (hITF).[16] In normal gastric mucosa, TFF1 is mainly expressed in the foveolar and superficial epithelium, whereas TFF2 is seen in mucous glands.[15,16] Mucins are large-molecular-weight glycoproteins synthesized by secretory epithelial tissues as membrane-bound or secreted proteins.[17] In normal gastric mucosa, MUC5AC co-localizes with TFF1 in the surface/foveolar epithelium and MUC6 is expressed, like TFF2, in the mucous cells of the neck zone and in the antral glands.[15,18–20] MUC1 is expressed in foveolar epithelium and oxyntic glands.[21,22] The intestinal mucin MUC2 is expressed in the Golgi region of foveolar cells in normal gastric antral mucosa and is expressed *de novo* in goblet cells (GC) of IM.[18,23,24]

In the present study we evaluated the expression of trefoil peptides (TFF1 and TFF2) and mucins (MUC1, MUC2, MUC5AC, and MUC6) in a series of 29 hyperplastic and adenomatous polyps of the stomach, eight of which displayed foci of malignant transformation. The aim of the study was two-fold: to characterize the expression profile of trefoil peptides and mucins on each

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type of gastric polyp and to investigate any modification of the profile associated with malignant transformation.

MATERIALS AND METHODS

Tissue material

Twenty-nine gastric polyps (10 hyperplastic and 19 adenomatous), examined at Hospital S. João/Medical Faculty and IPATIMUP, Porto, Portugal and the Medical Faculty of UFMG, Belo Horizonte, Brazil, were studied. Eight samples displayed malignant transformation: one hyperplastic and seven adenomatous polyps. Tissue fragments were fixed in 10 per cent formaldehyde and embedded in paraffin. Serial sections of 4 μm were stained with haematoxylin and eosin (H&E) and by immunohistochemistry.

Histological study

The presence of GC, intestinal-type absorptive cells (AC), and Paneth cells (PC) was evaluated in each lesion. Adenomatous polyps were classified into two groups, tubular and villous, including in the latter those with a tubulo-villous structure. Malignant foci were classified according to Laurén as intestinal- and diffuse-type carcinoma.[25]

Immunohistochemistry

A modification of the avidin–biotin–peroxidase method was applied[26] with 3,3′-diaminobenzidine as the chromogen. Sections were incubated overnight at 4°C with monoclonal antibodies against TFF1 (BC4, diluted 1:4; Cis Bio International, Gif-Sur-Yvette, France), TFF2 (diluted 1:4; kindly provided by Dr G. Elia), MUC1 (HMFG-1, diluted 1:100; Immunotech, Marseille, France),[22] MUC2 (PMH1, undiluted),[24] MUC5AC (CLH2, diluted 1:1000; Chemicon International Inc., Temecula, CA, U.S.A.),[19] and MUC6 (CLH5, diluted 1:2; kindly provided by Drs C. Reis and H. Clausen). All series included positive controls; negative controls were performed by replacement of the primary antibodies with immunoglobulins of the same class and concentration.

Scoring of immunoreactivity

Immunoreactivity was scored according to the presence of immunoreactive cells: −, none or rare positive cells (<5 per cent); +, 5–25 per cent; ++, 25–75 per cent; +++, >75 per cent.

RT-PCR study

Semi-quantitative RT-PCR studies were performed in formalin-fixed, paraffin-embedded material from six hyperplastic and four adenomatous polyps. Microdissection of the lesions was performed in four serial sections of 10 μm each, selected by examination of a consecutive H&E section. The material was incubated with xylol (5 min), ethanol (5 min), and acetone (1 min), being recovered by centrifugation at 13 000 *g* (1 min), after each incubation step. Total RNA was extracted as previously described.[27] First-strand synthesis was made by random 6-mer priming using M-MLV reverse transcriptase at 42°C for 15 min in the presence of 7 mM $MgCl_2$. For PCR, we used Taq polymerase in the presence of 2 mM $MgCl_2$. Semi-quantitation of TFF1 and TFF2 mRNA expression levels was performed by co-amplification of the target gene (TFF1 or TFF2) and the housekeeping gene GAPDH, and correction of expression levels relative to GAPDH levels. For each sample, the PCR bands resulting from co-amplification of TFF1 or TFF2 and GAPDH were quantified separately using the computer software Multi-Analyst/PC (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Relative expression levels of both TFF1 and TFF2 were defined as percentage ratios of TFF1/GAPDH and TFF2/GAPDH, respectively. Specific primers for TFF1 (5′-TTTGGAGCAGAGAGGAGGCAATG and 5′-ACCACAATTCTGTCTTTCACGGGG), TFF2 (5′-AGTTGGAGAAGCAGCACTTCC and 5′-GGATCAGTGCGTCATGGAG), and GAPDH (5′-ACCCAGAAGACTGTGGATGG and 5′-GGATGACCTTGCCCACAG) were chosen. Cycling conditions were 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. After determination of the PCR exponential phase for the three amplicons, PCRs were amplified for 25 cycles. Negative controls were performed by replacement of the template DNA with water. As positive controls, we used total RNA extracted from whole normal gastric mucosa for TFF1 and from the microdissected glandular zone for TFF2.

Pattern of cellular differentiation

Regarding TFF1 and MUC5AC expression and the presence of intestinal-type cells (GC, AC, and PC), three main phenotypes were defined: (1) complete gastric phenotype, with co-expression of TFF1 and MUC5AC and absence of intestinal-type cells; (2) incomplete gastric phenotype, with consistent expression of TFF1, no expression of MUC5AC, and very rare GC; (3) non-gastric (intestinal) phenotype, with no expression of TFF1 or MUC5AC, and with the presence of intestinal-type cells.

Statistical analysis

For statistical analysis, the chi-square test with Yates' correction was performed. Fisher's exact test was used whenever appropriate. Differences were taken to be significant at $p<0{\cdot}05$.

RESULTS

Histological analysis

In hyperplastic polyps, AC, PC, and IM were not observed. Foci of diffuse-type carcinoma were present in one of the ten hyperplastic polyps.

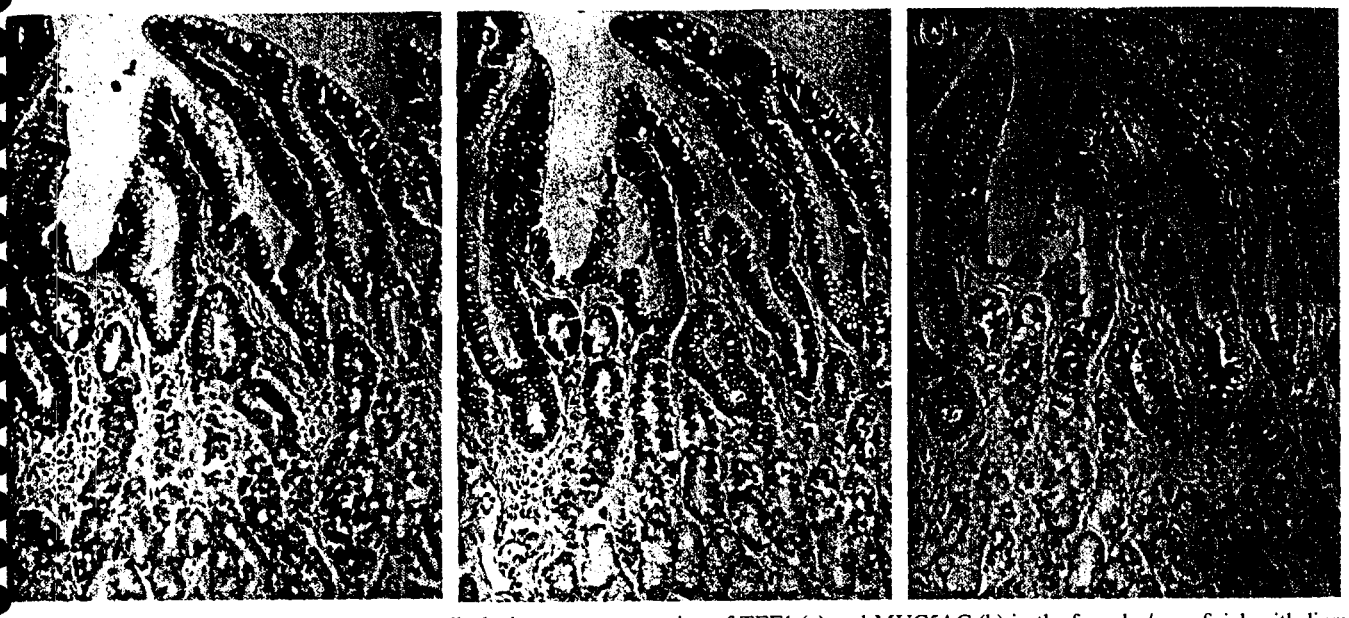


Fig. 1—Normal gastric mucosa of the antrum displaying strong expression of TFF1 (a) and MUC5AC (b) in the foveolar/superficial epithelium and cells of the neck zone. MUC1 is also expressed (c), though less intensely, in the surface epithelium and neck zone cells

In adenomatous polyps, villous or tubulo-villous architecture was observed in 12 cases (63·1 per cent). Foci of intestinal-type carcinoma were observed in seven adenomatous polyps (36·8 per cent). Carcinomatous foci were significantly more common in adenomas with villous features (7/12) than in those with tubular architecture (0/7) ($p=0·01$).

Expression of trefoil peptides and mucins

Normal gastric mucosa—TFF1 expression was seen in the cytoplasm of superficial/foveolar epithelium of antral and oxyntic mucosa and mucopeptic cells of the neck, and focally in antral glands. TFF2 expression was observed in the cytoplasm of mucopeptic cells of the neck zone, mucous glands of the antrum, and principal cells of the body. MUC1 expression was observed in superficial/foveolar and neck zone cells; the chief and parietal cells showed diffuse cytoplasmic staining or canalicular system staining, respectively. MUC2 expression was observed in the Golgi region of some foveolar cells of the normal antral mucosa. MUC5AC was observed in the perinuclear region in superficial/foveolar cells and mucopeptic cells of the neck region. MUC6 expression was observed in mucopeptic cells of the neck zone and in mucous glands of the antrum. Figure 1 shows the pattern of expression of TFF1 (a), MUC5AC (b), and MUC1 (c) in normal gastric mucosa.

Gastric polyps—Hyperplastic polyps: TFF1 and MUC5AC were detected in every case in the cytoplasm of more than 75 per cent of the cells (Figs 2a and 2b). MUC1 was also expressed in every hyperplastic polyp in more than 75 per cent of the cells, both in the cytoplasm and at the cell membrane (Fig. 2c). Two cases presented MUC6 expression in fewer than 25 per cent of the cells. MUC2 expression was detected, focally, in another two cases. TFF2 expression was not observed in any case (Table I).

Adenomatous polyps: TFF1 expression was observed in 16 cases (84·2 per cent). MUC1 was detected in 14 cases (73·7 per cent), predominantly at the apical membrane and, focally, in the cytoplasm. All but one case expressing TFF1 also exhibited MUC1 immunoreactivity. MUC2 expression was detected in seven cases (36·8 per cent). No relationship was observed between MUC2 immunoreactivity and expression of TFF1 or other mucins. MUC5AC expression was observed in nine cases (47·4 per cent), all of them co-expressing TFF1 and MUC1. MUC6 was expressed in seven cases (36·8 per cent), all of them displaying also TFF1, MUC1, and MUC5AC immunoreactivity. TFF2 expression was not detected in any case (Table I).

Pattern of cell differentiation—All hyperplastic polyps exhibited a complete gastric phenotype (Table II). In adenomatous polyps, three main phenotypes could be identified: complete gastric phenotype (high expression of TFF1 and MUC5AC)—nine cases (47·4 per cent) (Fig. 3); incomplete gastric phenotype (consistent expression of TFF1, no expression of MUC5AC, and rare GC)—seven cases (36·8 per cent); non-gastric (intestinal) phenotype (lack of expression of both TFF1 and MUC5AC)—3 cases (15·8 per cent) (Fig. 4) (Table II).

RT-PCR—In hyperplastic polyps, we detected high levels of TFF1 mRNA expression as evaluated by semi-quantitative RT-PCR, ranging from 115·5 to 861·1 per cent (expressed as a percentage of GAPDH expression). In the adenomatous polyps, TFF1 relative expression levels ranged from 33·1 to 257·6 per cent (Table III and Fig. 5). TFF2 mRNA was not detected in any of the ten samples of gastric polyps. In normal gastric mucosa

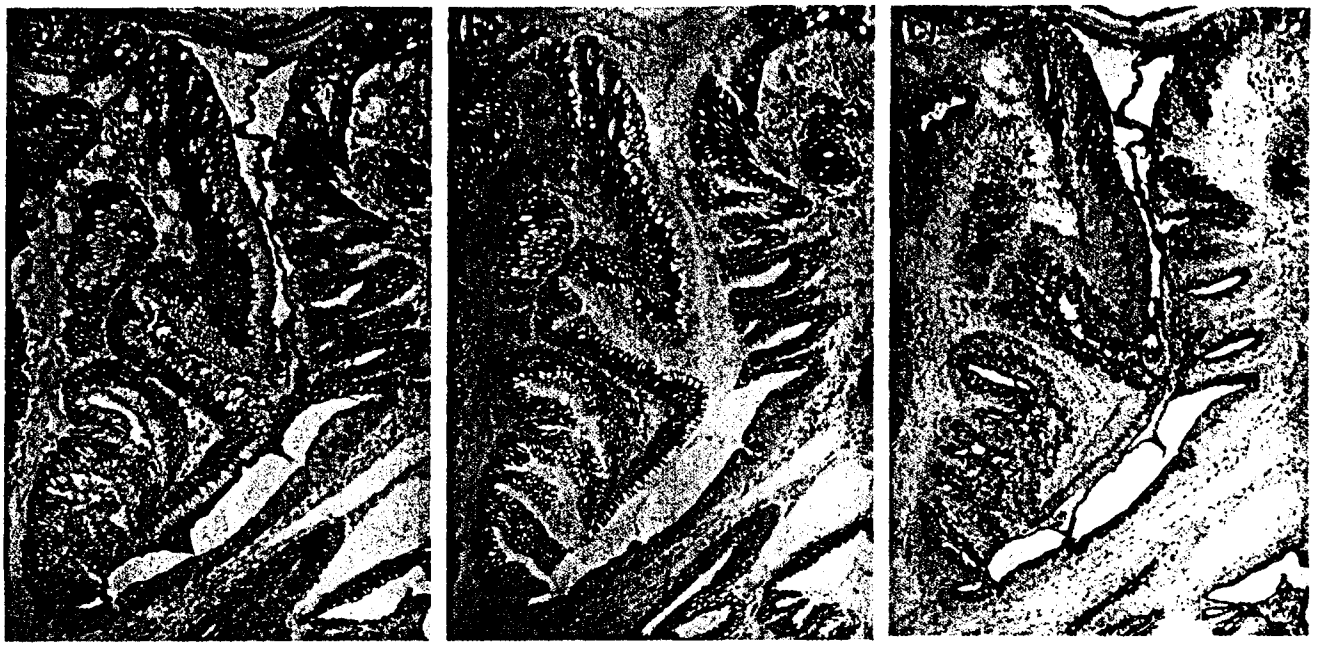


Fig. 2—Hyperplastic polyp displaying the complete gastric phenotype: immunoreactivity for TFF1 (a) and MUC5AC (b) is observed in almost every cell and co-localizes with MUC1 (c)

Table I—Pattern of expression of trefoil peptides and mucins in hyperplastic and adenomatous polyps

	Expression of trefoil peptides and mucins No. of positive cases (%)					
Polyps	TFF1	TFF2	MUC1	MUC2	MUC5AC	MUC6
Hyperplastic (n=10)	10 (100%)	0 (0%)	10 (100%)	2 (20%)	10 (100%)	2 (20%)
Adenomatous (n=19)	16 (84·2%)	0 (0%)	14 (73·7%)	7 (36·8%)	9 (47·4%)	7 (36·8%)

Table II—Pattern of cellular differentiation in hyperplastic and adenomatous polyps

	Phenotype*		
Polyps	Complete gastric n (%)	Incomplete gastric n (%)	Non-gastric (intestinal) n (%)
Hyperplastic (n=10)	10 (100%)	0 (0%)	0 (0%)
Adenomatous (n=19)	9 (47·4%)	7 (36·8%)	3 (15·8%)

*See text for definitions.

used as a positive control, both TFF1 and TFF2 mRNAs were detected (data not shown).

The expression data yielded by immunohistochemistry and by RT-PCR showed a good correlation for TFF1 in every case (Table III). All cases were negative for TFF2 expression, both at the mRNA and at the protein level.

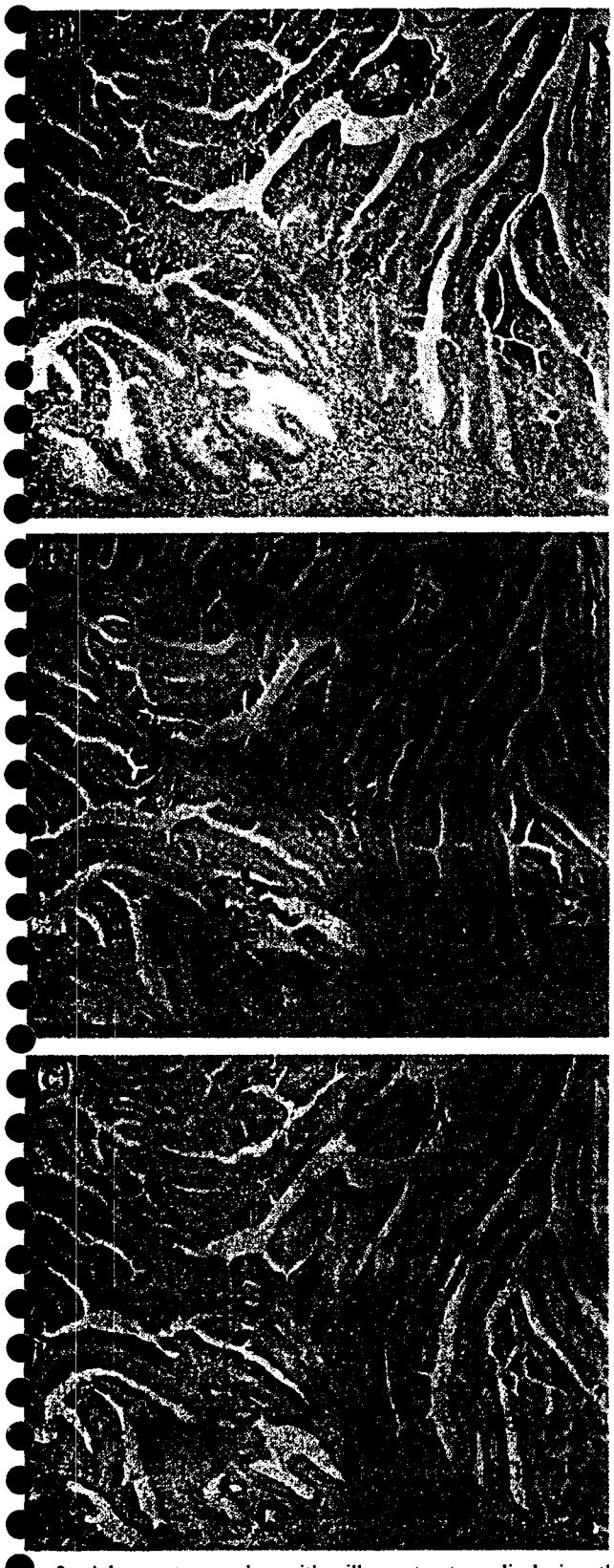
Relationship between architecture and immunohistochemistry

We observed a significant relationship between villous architecture of the polyps and MUC1 expression (p<0·04) as well as between villous architecture and signs of (complete or incomplete) gastric differentiation (p<0·04).

Malignant transformation

Foci of malignant transformation were observed in eight cases: a diffuse carcinoma occurring in a hyperplastic polyp and seven intestinal-type carcinomas occurring in adenomatous polyps.

Signs of complete gastric differentiation were observed in the hyperplastic polyp and the corresponding diffuse carcinoma. MUC6 was observed in the neoplastic cells of this case, despite being undetectable in the hyperplastic cells of the polyp (Table IV).



g. 3—Adenomatous polyp with villous structure displaying the complete gastric phenotype, characterized by co-localization of TFF1 and MUC5AC (b). (c) Immunoreactivity for MUC1 is observed in the apical region and membrane in more than 50 per cent of the cells

Five adenomatous polyps with malignant transformation exhibited signs of complete gastric differentiation in both components. In the remaining two cases, the adenomatous epithelium displayed signs of incomplete gastric differentiation, whereas in the corresponding carcinomas, both TFF1 and MUC5AC were expressed (Table IV).

MUC1 was detected in both the non-malignant and the malignant components of every hyperplastic and adenomatous polyp with malignant transformation. MUC2 and MUC6 were irregularly expressed in the hyperplastic and adenomatous polyps and corresponding malignant foci (Table IV).

The relationship between malignancy and morphological and/or immunohistochemical features was stronger for villous architecture ($p<0{\cdot}02$) than for MUC1 expression ($p=0{\cdot}11$) or gastric phenotype ($p=0{\cdot}26$).

DISCUSSION

We undertook the present study in order to characterize the patterns of cell differentiation in hyperplastic and adenomatous polyps of the stomach, with and without malignant transformation. For this purpose, we evaluated the expression of trefoil peptides (TFF1 and TFF2) and mucins (MUC1, MUC2, MUC5AC, and MUC6). In agreement with data on record,[14,15,19] we observed co-expression of TFF1 and MUC5AC in the foveolar/superficial epithelium of gastric mucosa. MUC1 was also expressed in the foveolar/superficial epithelium and neck zone cells. In keeping with data on record,[15] immunohistochemical expression of TFF2 was not observed at the surface epithelium, although the expression of the corresponding mRNA has been previously reported by *in situ* hybridization.[15] In the mucous cells of the antrum, the chief cells of the body, and neck zone cells, we observed, as have others, expression of both TFF2 and MUC6.[13,15,16,18,21] In normal gastric mucosa, immunoreactivity for MUC2 was restricted to the Golgi region of some foveolar cells of the antrum. Altogether these results show that trefoil peptides and mucins are highly regulated in gastric mucosa, being expressed in a cell-specific manner.[15–18,21] Based on the pattern of expression of TFF1 and MUC5AC and on the cell types observed by conventional histology, we identified three types of differentiation: complete gastric, incomplete gastric, and non-gastric (intestinal) differentiation. The definition of the incomplete gastric phenotype was based on the lack of MUC5AC expression and the consistent expression of TFF1. Since TFF1 expression was previously shown in the distal colon,[28] we cannot exclude the possibility that some of the cases expressing TFF1 alone may have a mixed (gastric/intestinal) phenotype.

In hyperplastic polyps, we observed co-expression of TFF1 and MUC5AC, similar to the pattern observed in normal gastric mucosa. MUC1 was also observed in a high percentage of cells, whereas TFF2 and MUC6 were not detected. These findings reinforce the concept that hyperplastic polyps represent an overgrowth of the

Fig. 4—Adenomatous polyp displaying the non-gastric phenotype: the tubular structures of the lesion do not display TFF1 (a) or MUC5AC (b) immunoreactivity, in contrast with the intense immunostaining observed in the adjacent gastric mucosa. (c) Immunoreactivity for MUC1 is observed at the membrane of every cell of the adenomatous lesion (arrows)

superficial zone of gastric mucosa.[2] The level of expression of trefoil peptides (TFF1 and TFF2) as evaluated by immunohistochemistry was in keeping with the expression of their RNAs as determined by RT-PCR: high expression of TFF1 and no expression of TFF2. It is tempting to suggest that the latter result probably reflects the low sensitivity of the method that we employed, using paraffin-embedded material, since one would expect some level of TFF2 mRNA expression in polyps originating in the surface epithelium.[15]

In adenomatous polyps, the pattern of expression of trefoil peptides and mucins was heterogeneous. Complete gastric differentiation was detected in 47·4 per cent of the cases, incomplete gastric differentiation in 36·8 per cent, and no gastric differentiation in 15·8 per cent. Our results reinforce data on record showing that adenomatous polyps may exhibit different types of cell differentiation, gastric-, mixed- or intestinal-types.[2,7,11] The study of TFF1 both by immunohistochemistry and by RT-PCR also showed the heterogeneity of the series of adenomatous polyps (Table III).

A significant relationship was found between villous structure and maligant transformation in adenomatous polyps, in keeping with previous reports.[1,2,6]

Kushima *et al.*[7] showed that in adenomas with intestinal-type differentiation, the immunoexpression of p53 was higher than in adenomas with gastric-type differentiation. In a preliminary study on p53 expression in a series of gastric polyps, we did not find a relationship between immunoreactivity for p53 and the

Table III—Comparison of TFF1 expression as evaluated by semi-quantitative RT-PCR and immunohistochemistry in ten gastric polyps

Case No.	Polyp type	RT-PCR (%)	IHC (% cells)
1	H	486·7	>75
4	H	748·3	>75
5	H	350·9	>75
6	H	861·1	>75
7	H	364·7	>75
8	H	115·5	>75
16	A	40·5	<25
17	A	38·3	<25
21	A	33·1	<25
29	A	257·6	>75

A=adenomatous; H=hyperplastic; IHC=immunohistochemistry.

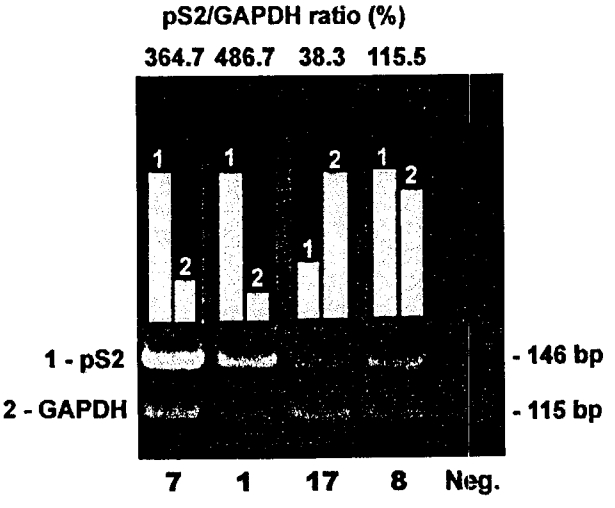


Fig. 5—TFF1 expression evaluated by semi-quantitative RT-PCR. Co-amplification of TFF1 (146 bp) and GAPDH (115 bp) analysed on a 5 per cent PAA gel stained with ethidium bromide. Bars 1 and 2 represent band intensities of TFF1 and GAPDH, respectively. Expression levels of TFF1 were calculated as a TFF1/GAPDH ratio. Case numbers refer to those in Table III. Neg=negative control

Table IV—Pattern of expression of trefoil peptides and mucins in polyps and corresponding malignant foci

Cases	Phenotype*	TFF1	TFF2	MUC1	MUC2	MUC5AC	MUC6
Case 10							
Hyperplastic epithelium	Complete gastric	+++	–	+++	–	+++	–
Diffuse-type carcinoma	Complete gastric	+	–	++	–	+	+
Case 14							
Adenomatous epithelium	Incomplete gastric	+	–	++	–	–	–
Intestinal-type carcinoma	Complete gastric	+	–	+++	†	+	–
Case 18							
Adenomatous epithelium	Incomplete gastric	++	–	++	+	–	–
Intestinal-type carcinoma	Complete gastric	+	–	+++	–	+	–
Case 22							
Adenomatous epithelium	Complete gastric	+	–	+++	†	+++	++
Intestinal-type carcinoma	Complete gastric	+	–	+++	–	+	+
Case 25							
Adenomatous epithelium	Complete gastric	++	–	+++	–	+++	+
Intestinal-type carcinoma	Complete gastric	+	–	+++	–	+++	–
Case 26							
Adenomatous epithelium	Complete gastric	++	–	++	–	++	+
Intestinal-type carcinoma	Complete gastric	++	–	++	–	++	–
Case 27							
Adenomatous epithelium	Complete gastric	++	–	+++	–	++	–
Intestinal-type carcinoma	Complete gastric	++	–	+++	–	++	–
Case 28							
Adenomatous epithelium	Complete gastric	+++	–	++	–	+++	+
Intestinal-type carcinoma	Complete gastric	+++	–	+	–	+++	+

*See text for definitions and scoring of immunoreactivity.
†Golgi staining in foveolar-like cells as sometimes observed in normal gastric mucosa.

phenotype of the lesions or histological features of aggressiveness, such as the type of structure and the grade of dysplasia (data not shown). The discrepancies between our findings and those reported by Kushima *et al.*[7] may be due to the different criteria used in the two series for the characterization of cell differentiation. One cannot exclude the possibility that cases considered by Kushima *et al.*[7] as having an intestinal phenotype might have been classified as incomplete gastric differentiation according to the criteria and markers used in the present study.

In the present series, all polyps with foci of malignant transformation exhibited signs of gastric differentiation: complete-type in one hyperplastic polyp and in five adenomatous polyps, and incomplete-type in two adenomatous polyps.

Every carcinoma developing in a gastric polyp corresponded to carcinomas in the early stages of development and exhibited expression of TFF1 and MUC5AC, which are usually considered markers of gastric differentiation.[14,19] These findings are in keeping with those reported by Reis *et al.*[19] and Ho *et al.*,[21] showing that gastric differentiation is a common event in the first stages of malignant transformation in the stomach. Our findings show that a subset of the so-called 'intestinal' carcinomas of the stomach exhibits signs of gastric differentiation at the cellular level,[19] thus supporting our contention that they should be designated as 'glandular' rather than 'intestinal' carcinomas.[29]

MUC1 was detected in all hyperplastic polyps, in the cytoplasm and at the cell membrane, in 14 adenomatous polyps (73·7 per cent), and in every carcinoma. In adenomatous and carcinomatous lesions, immunoreactivity was predominantly expressed at the cell membrane. For practical purposes, the evaluation of MUC1 expression should not be dissociated from the architecture of adenomatous polyps. In fact, every adenomatous polyp with malignant transformation had villous architecture and displayed MUC1 immunoreactivity, whereas the single villous adenomatous polyp without MUC1 expression did not display malignancy. These observations should not, however, obscure the absence of malignant foci in tubular adenomatous polyps, regardless of the presence or absence of MUC1 expression. A larger series is necessary to elucidate the putative value of MUC1 expression as a predictor of malignant transformation in adenomatous polyps.

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ANNEX

Fig. 1

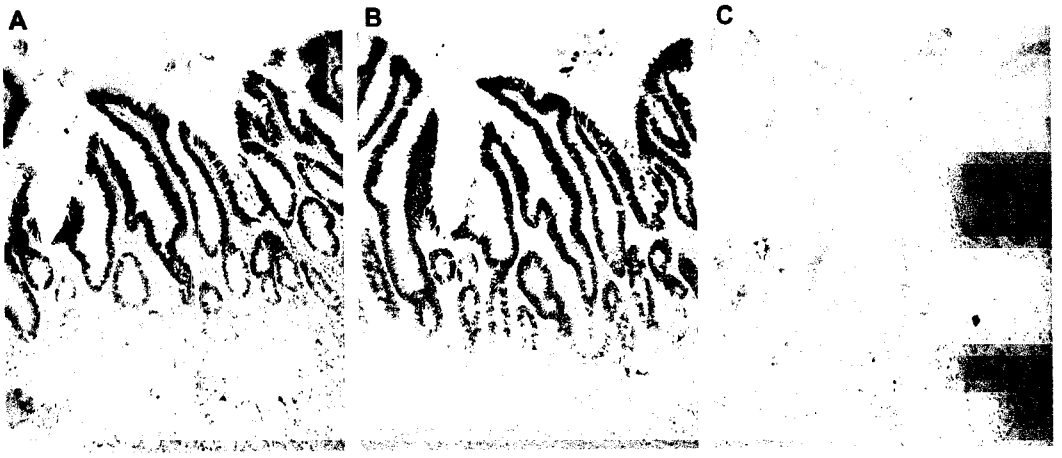


Fig.2

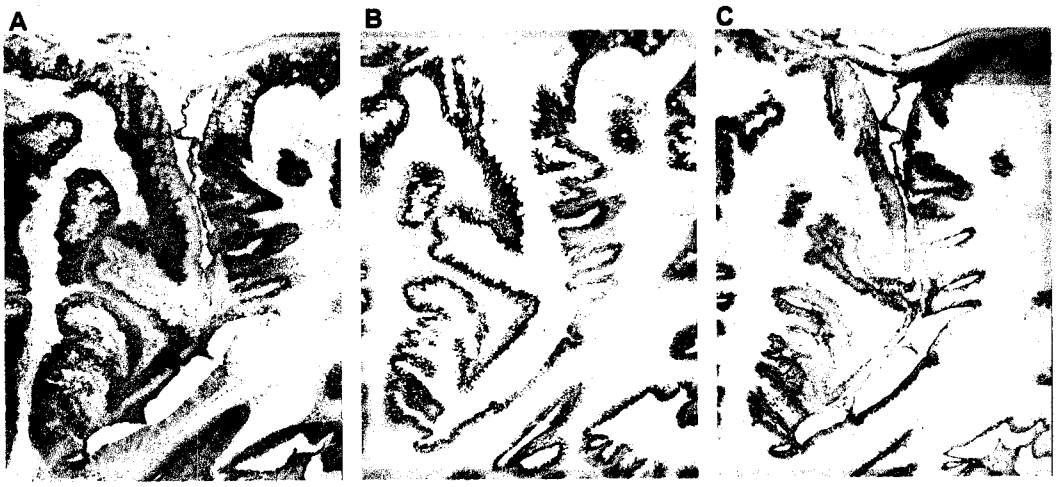


Fig.3

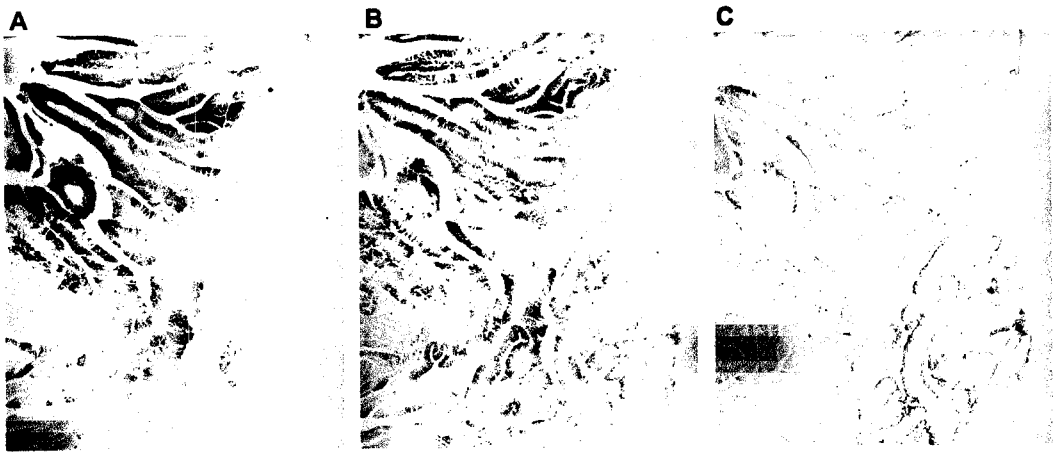
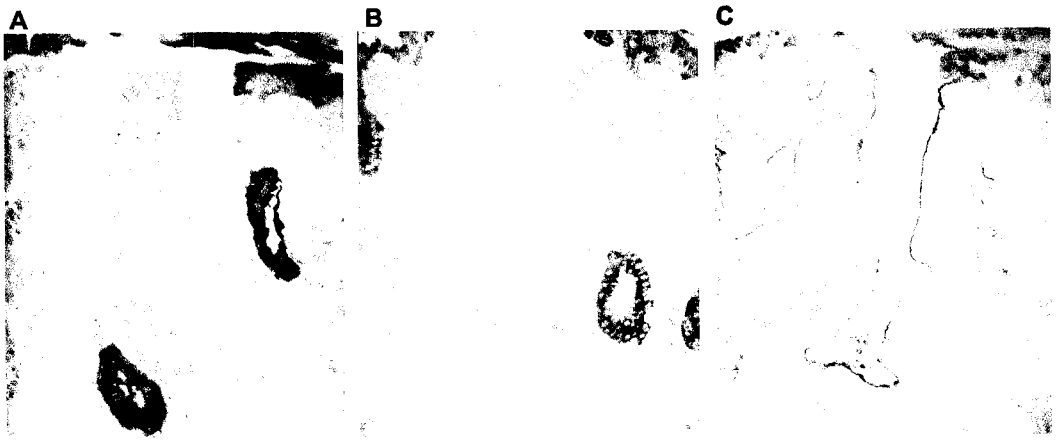


Fig.4



PAPER IV

Title: Gastric carcinoma exhibits distinct types of cell differentiation: An immunohistochemical study of trefoil peptides (TFF1 and TFF2) and mucins (MUC1, MUC2, MUC5AC and MUC6).

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Running title: Cell differentiation and expression of trefoil peptides and mucins in gastric carcinoma.

SUMMARY

We have previously described the expression of trefoil peptides (TFF1 and TFF2) and mucins (MUC1, MUC2, MUC5AC and MUC6) in gastric polyps. In the present study we characterised the expression profile of the aforementioned trefoil peptides and mucins in 96 gastric carcinomas, in an attempt to further the understanding of the histogenesis and cell differentiation of gastric carcinoma. Taking together the co-expression of trefoil peptides and mucins, three phenotypes were defined: complete gastric; incomplete gastric; and non-gastric phenotype. Gastric differentiation (complete and incomplete) was observed in 30 out of 33 (90.9%) diffuse carcinomas and in 38 out of 53 (71.7%) intestinal carcinomas. Non-gastric differentiation was observed only in three (9.1%) diffuse carcinomas and in 15 (28.3%) intestinal carcinomas. The phenotypes observed in intestinal carcinomas were similar to those previously observed in adenomatous polyps whereas most diffuse carcinomas mimic the phenotype of hyperplastic polyps. The percentage of cases displaying non-gastric phenotype was higher, though not significantly, in tumours that had invaded the gastric wall than in T1 tumours, regardless of histotype. We conclude that gastric-type differentiation is retained in the majority of gastric carcinomas, being more prominent in diffuse than in intestinal carcinomas, and in early than advanced carcinomas (we still ignore if the trend towards loss of gastric differentiation along tumour progression is paralleled by intestinalisation of the carcinomas).

Key-words: adenomatous polyps, hyperplastic polyps, gastric carcinoma, mucins, stomach, trefoil peptides.

INTRODUCTION

Studies based on the immunohistochemical expression of markers of cell differentiation[1-7] and on electron microscopic features of neoplastic cells,[6,8,9] suggest that two main types of cell differentiation — intestinal and gastric — can be recognised in gastric carcinoma.

Trefoil peptides (TFF peptides) constitute a group of small secretory peptides bearing one or more trefoil structural motifs (P-domains).[10,11] The expression of these peptides was shown to be closely related to mucous secreting gastrointestinal epithelia.[10-12]

Mucins are large molecular-weight glycoproteins synthesised by secretory epithelial tissues as membrane-bound or secreted glycoproteins[13] and are expressed in a cell- and tissue-specific pattern.[14,15]

In normal gastric mucosa, TFF1 (pS2) co-localises with MUC5AC in the surface/foveolar epithelium and TFF2 (hSP) is expressed together with MUC6 in the mucous cells of the neck zone of the oxyntic mucosa and in the antral glands.[15-18] MUC1, which is a membrane-bound mucin, is expressed in the foveolar epithelium of gastric antrum and oxyntic glands of the gastric body.[14,19] The intestinal mucin MUC2 is usually not expressed in gastric mucosa.[14,20,21]

In previous studies we have analysed the expression of trefoil peptides and mucins in pre-malignant lesions of the stomach.[17,18,22] In incomplete intestinal metaplasia (types II and III) a mixed gastric and intestinal phenotype was observed, while complete, type I intestinal metaplasia was characterised by a shift from gastric-type to intestinal-type phenotype.[17] In gastric polyps, we observed that gastric-type differentiation is a feature of hyperplastic polyps and of a subset of adenomatous polyps which is shared by early carcinomas arising in some of these polyps.[18,22]

In the present study we evaluated the concurrent expression of two trefoil peptides (TFF1 and TFF2) and four mucins (MUC1, MUC2, MUC5AC and MUC6) in a series of 96 gastric carcinomas. We aimed to characterise the expression profile of trefoil peptides and mucins in gastric carcinoma *per se* and in comparison with the profiles previously observed in premalignant lesions of the stomach,[18] in an attempt to further the understanding of the histogenesis and cell differentiation of gastric carcinoma.

MATERIALS AND METHODS

Tissue material

Surgical specimens from 96 gastric carcinomas resected and diagnosed at Hospital S. João/Medical Faculty and IPATIMUP, Porto, Portugal and Medical Faculty of UFMG, Belo Horizonte, Brazil, were studied. Tissue fragments were fixed in 10% formaldehyde and embedded in paraffin. Serial sections of 4μm were obtained from each block and used for routine staining with haematoxylin and eosin and immunohistochemistry (IHC).

Gastric carcinomas were classified according to Laurén[23] as intestinal (n=53), diffuse (n=33) and unclassifiable (n=10). Regarding the depth of invasion of the gastric wall, the cases were classified as: early, corresponding to T1 (n=13); and advanced (n=83), corresponding to T2 (n=16), T3 (n=44) and T4 (n=23).

Immunohistochemistry

A modification of the avidin-biotin-peroxidase method was applied with 3,3'-diaminobenzidine as chromogen. Sections were incubated overnight at 4^0C with monoclonal antibodies (MoAbs) against TFF1 (GE1,[24] diluted 1:1200), TFF2 (hSP,[25] diluted 1:4), MUC1 (HMFG-1, diluted 1:100, Immunotech, Marseille, France), MUC2 (PMH1,[20] undiluted), MUC5AC (CLH2, diluted 1:1000, Chemicon International Inc., Temecula, CA, USA) and MUC6 (CLH5,[17] diluted 1:2). All series included positive controls; negative controls were performed by substitution of the primary MoAbs with immunoglobulins of the same class and concentration. A case was considered positive whenever more than 5% of cells displayed immunohistochemical staining, irrespective of its intensity and localisation.

Pattern of cell differentiation (Phenotype)

The pattern of cell differentiation was defined according to the criteria previously described by Nogueira *et al.*[18] and based upon the expression of trefoil peptides and mucins typically expressed in the superficial zone of normal gastric mucosa. Three main phenotypes were defined: 1) complete gastric phenotype — co-expression of TFF1 and MUC5AC regardless of the expression of TFF2 and MUC6; 2) incomplete gastric

phenotype — expression of either TFF1 or MUC5AC regardless of the expression of TFF2 and MUC6; 3) non-gastric phenotype — no expression of TFF1, TFF2, MUC5AC and MUC6.

Statistical analysis

The statistical analysis of the results was performed using the Pearson-χ^2 test. Differences were considered to be significant at $p<0.05$ and suggestive at $p<0.1$.

RESULTS

Normal gastric mucosa

There was co-expression of TFF1 and MUC5AC in the cytoplasm of superficial and foveolar epithelium of antral and oxyntic mucosa as well as in mucopeptic cells of the neck; TFF1 was also observed focally in antrum glands. In the cytoplasm of mucopeptic cells of the neck zone, mucous glands of the antrum, and principal cells of the body there was co-expression of TFF2 and MUC6. MUC1 expression was observed in superficial/foveolar and neck zone cells; the principal and parietal cells showed a diffuse cytoplasmic staining or canalicular system staining, respectively. MUC2 expression was observed in the supranuclear Golgi region of rare foveolar cells of the antrum.

Gastric carcinoma

TFF1 and TFF2 expression was observed, respectively, in 64 cases (66.7%) and in 10 cases (10.4%) of the 96 gastric carcinomas (Table I). Immunoreactivity for MUC5AC was detected in 61 cases (63.5%) and MUC6 immunoexpression was found in 36 cases (37.5%) (Table I). TFF1, TFF2, MUC5AC and MUC6 antigens displayed an immunoexpression pattern characterised by diffuse cytoplasmic staining. MUC1 was detected in 79 cases (82.3%): predominantly at the apical membrane in intestinal-type tumours; and with a diffuse pattern of staining in the cytoplasm of the diffuse-type tumours. MUC2 immunoreactivity was detected in 22 cases (22.9%), in the cytoplasm of neoplastic cells.

In the whole series there was a significant correlation between TFF1 and MUC5AC expression ($p<0.0001$): 50 out of 64 cases positive for TFF1 (78.1%) also displayed immunoreactivity for MUC5AC; 21 out of 32 cases without expression of TFF1 (65.6%) were also negative for MUC5AC expression. No significant correlation was found between the immunohistochemical expression of TFF2 and MUC6: six out of 10 cases positive for TFF2 (60.0%) were positive for MUC6; 56 out of 86 cases without expression of TFF2 (65.1%) were also negative for MUC6 expression.

Table I - Comparison between the immunoexpression pattern of trefoil peptides and mucins and the histological type of 96 gastric carcinomas.

Histological type	IHC positive cases (%)					
	TFF1	MUC5AC	TFF2	MUC6	MUC1	MUC2
Diffuse ca (n = 33)	25 (75.8)	23 (69.7)	5 (15.2)	8 (24.2)	24 (72.7)	8 (24.2)
Intestinal ca (n = 53)	30 (56.6)	29 (54.7)	4 (7.5)	22 (41.5)	46 (86.8)	11 (20.8)
Unclassifiable ca (n = 10)	9 (90.0)	9 (90.0)	1 (10.0)	6 (60.0)	9 (90.0)	3 (30.0)
p value	0.05	0.07	0.53	0.10	0.20	0.80
Total (n = 96)	64 (66.7)	61 (63.5)	10 (10.4)	36 (37.5)	79 (82.3)	22 (22.9)

The comparison between the immunohistochemical expression of the antigens under study and the histological classification of the tumours revealed a significant association (p=0.05) between the expression of TFF1 and the diffuse and unclassifiable histological types (Table I). A suggestive association (p=0.07) was observed between MUC5AC expression and the diffuse and unclassifiable histotypes (Table I). No relationship was found between the expression of TFF2, MUC1, MUC2 or MUC6 and the histotype of the tumours.

According to the expression pattern of trefoil peptides and mucins, 50 cases (52.1%) presenting co-expression of TFF1 and MUC5AC were identified as complete gastric-type (Table II) (Figs. 1 and 2). Incomplete gastric phenotype was observed in 28 cases (29.2%) (Table II); three cases negative for TFF1, TFF2 and MUC5AC but expressing MUC6 were included in the group of incomplete gastric-type differentiation. Lack of expression of TFF1, TFF2, MUC5AC and MUC6 was found in the remaining 18 cases (18.7%), therefore identified as non-gastric phenotype (Table II) (Fig. 3).

Table II – Comparison between phenotypic differentiation and histological type of gastric carcinomas.

Histological type		Phenotype (%)			
	Cases (%)	Complete Gastric	Incomplete gastric	Non-gastric	p value
Diffuse ca	33 (34.4)	18 (54.5)	12 (36.4)	3 (9.1)	
Intestinal ca	53 (55.2)	24 (45.3)	14 (26.4)	15 (28.3)	0.06
Unclassifiable ca	10 (10.4)	8 (80.0)	2 (20.0)	0 (0)	
Total	96 (100)	50 (52.1)	28 (29.2)	18 (18.7)	

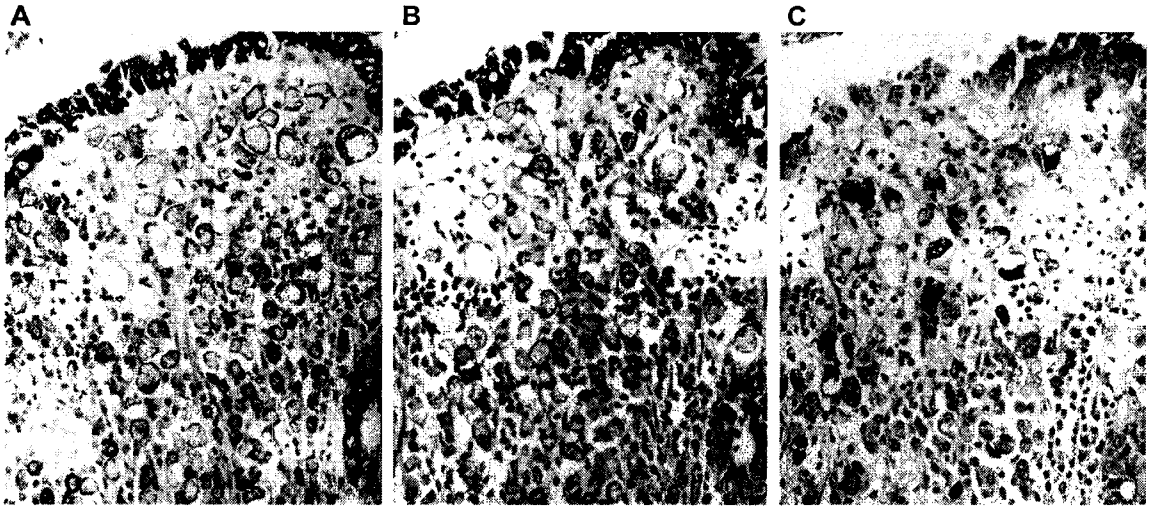


Fig. 1 - Diffuse gastric carcinoma displaying complete gastric phenotype: TFF1 (A) and MUC5AC (B) immunostaining is observed in the cytoplasm of tumour cells, and co-localise with the expression of MUC1 (C). (X 840)

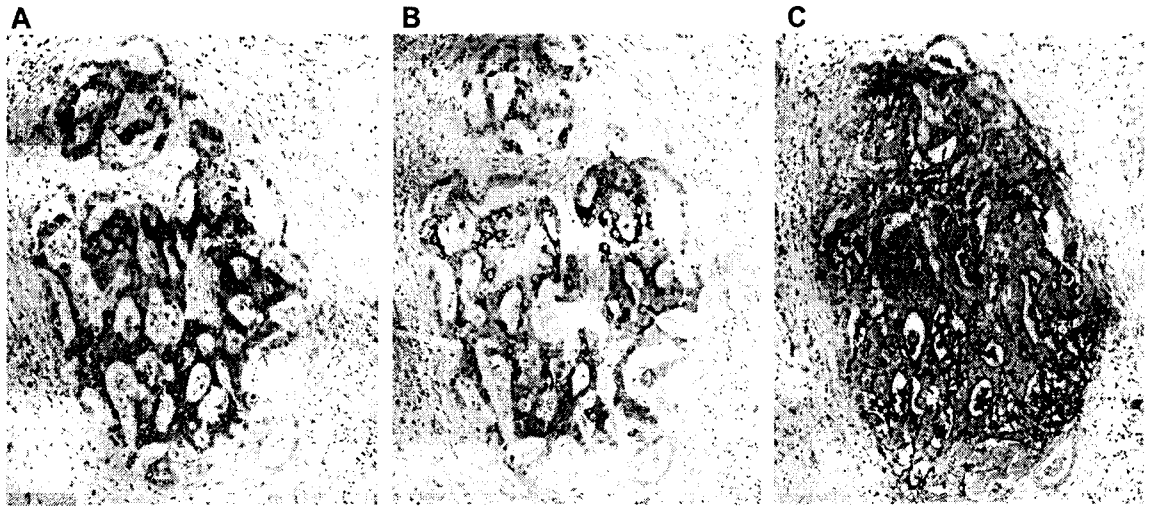


Fig. 2 - Intestinal gastric carcinoma presenting complete gastric-type differentiation characterised by the co-localisation of TFF1 and MUC5AC expression; MUC1 (C) is highly expressed in the same area. (X 420)

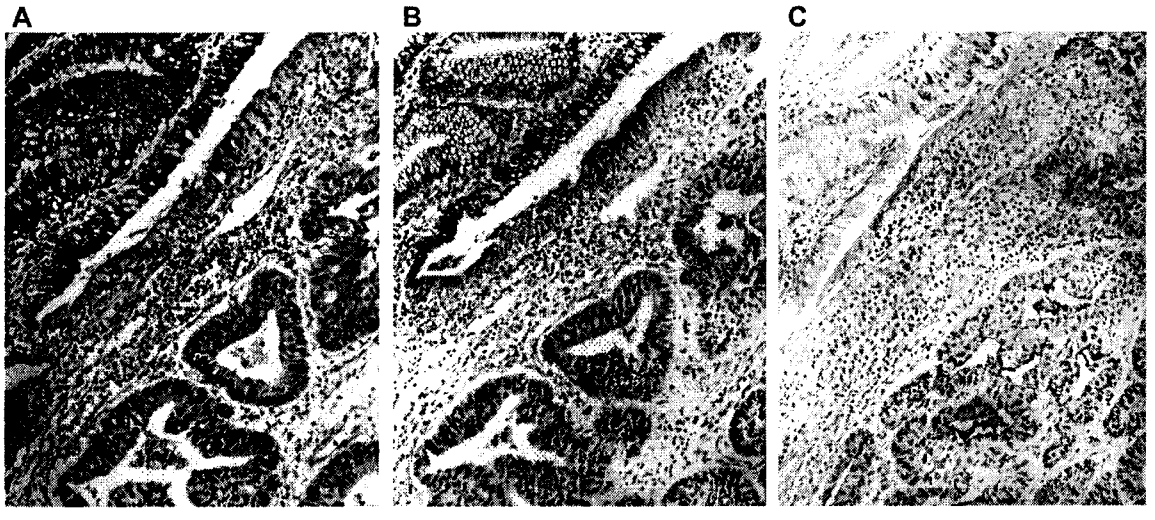


Fig. 3: Non-gastric phenotype in a gastric carcinoma of the intestinal histotype: no immunoreactivity is observed for TFF1 (A) and MUC5AC (B); MUC1 (C) expression is observed at the apical membrane of some neoplastic glands. (X 420)

The prevalence of MUC1 expression was similar in the three phenotypes: in 44 out of 50 cases (88.0%) with complete gastric-type differentiation; in 22 out of 28 cases

(78.6%) presenting incomplete gastric-type differentiation; and in 13 out of 18 cases (72.2%) classified as non-gastric phenotype. The same holds true regarding the expression of MUC2, which was seen in 14 out of 50 cases (28.0%) displaying complete gastric phenotype; in six out of 28 cases (21.4%) presenting incomplete gastric-type differentiation; and in two out of 18 cases (11.1%) with non-gastric phenotype.

The comparison between the histological type of the cases and the phenotype disclosed a suggestive association (p=0.06) between the diffuse and unclassifiable histotypes and the gastric (complete and incomplete) phenotypes, and between the intestinal histotype and the non-gastric phenotype (Table II).

Table III – Comparison between phenotypic differentiation and extent of wall invasion of gastric carcinomas, according to the histological type.

T stage	Cases	Phenotype (%)			
		Complete Gastric	Incomplete gastric	Non-gastric	p value
Diffuse					
Early	5	3 (60.0)	2 (40.0)	0	0.75
Advanced	28	15 (53.5)	10 (35.7)	3 (10.7)	
Intestinal					
Early	8	5 (62.5)	2 (25.0)	1 (12.5)	0.48
Advanced	45	19 (42.2)	12 (26.7)	14 (31.1)	
Total					
Early	13	8 (61.5)	4 (30.8)	1 (7.7)	0.53
Advanced	83	42 (50.6)	24 (28.9)	17 (20.5)	

Most of the early tumours were identified as complete gastric-type (8/13 – 61.5%) or incomplete gastric-type (4/13 – 30.8%) and only one early tumour, with intestinal histotype, was found to present a non-gastric phenotype (7.7%) (Table III); in contrast to this, in the group of advanced tumours, 17 out of 83 cases (20.5%) had a non-gastric phenotype (Table III); these differences did not attain, however, the threshold of statistical significance (p=0.53) (Table III). The same holds true regarding MUC2

expression, which was found to be more prevalent, though not significantly, in advanced tumours than in early tumours (p=0.16) (Table IV).

Table IV – Comparison between MUC2 expression and extent of wall invasion of gastric carcinomas according to the histological type.

T stage		MUC2 IHC (%)		
	Cases	**+**	**-**	**p value**
Diffuse				
Early	5	0 (0)	5 (100)	0.17
Advanced	28	8 (28.6)	20 (71.4)	
Intestinal				
Early	8	1 (12.5)	7 (87.5)	0.53
Advanced	45	10 (22.2)	35 (77.8)	
Total				0.16
Early	13	1 (7.7)	12 (92.3)	
Advanced	83	21 (25.3)	62 (74.7)	

DISCUSSION

The expression of mucins and trefoil peptides follows a cell- and tissue-specific pattern in normal tissues.[14,15] In the stomach, there is co-expression of TFF1 and MUC5AC in the surface/foveolar epithelium and TFF2 is expressed together with MUC6 in the mucous cells of the neck zone of the oxyntic mucosa and in the antral glands.[15-18] These features are in keeping with the hypothesis that the expression of trefoil peptides is closely related to mucous secreting epithelia[10-12] and that TFF peptides and mucins may act in a synergistic manner to protect and reconstitute epithelial tissues.[26,27] Changes in the pattern of expression of trefoil peptides and mucins were previously described in premalignant and malignant lesions of the stomach.[15,17-19,22,28,29]

In the present study we observed that TFF1 and MUC5AC were similarly expressed in gastric carcinomas (66.7% and 63.5%, respectively). Co-expression of TFF1 and MUC5AC was detected in 50 of the 96 cases (52.1%). At variance with this, expression of TFF2 and MUC6 was observed in a much lower percentage of the cases (10.4% and 37.5%, respectively) and co-expression of TFF2 and MUC6 was detected only in six of the 96 cases (6.3%).

Based on the pattern of expression of the different mucins and trefoil peptides, and in accordance with previously reported criteria,[18] we defined three main phenotypes of cell differentiation: complete gastric, incomplete gastric and non-gastric phenotype.

Overall, our study shows that the majority of gastric carcinomas retain signs of complete (52.1%) or incomplete (29.2%) gastric-type differentiation. Such signs are more often exhibited in diffuse and unclassifiable carcinomas than in intestinal-type carcinomas. However, gastric-type differentiation was also observed in intestinal-type carcinomas, supporting our contention that gastric carcinomas forming glands should be designated as "glandular" instead of "intestinal" carcinomas, in order to avoid mixing structural with cell differentiation concepts.[6,30] In 18.7% of the cases, mostly of the intestinal histotype, we found no signs of gastric-type differentiation.

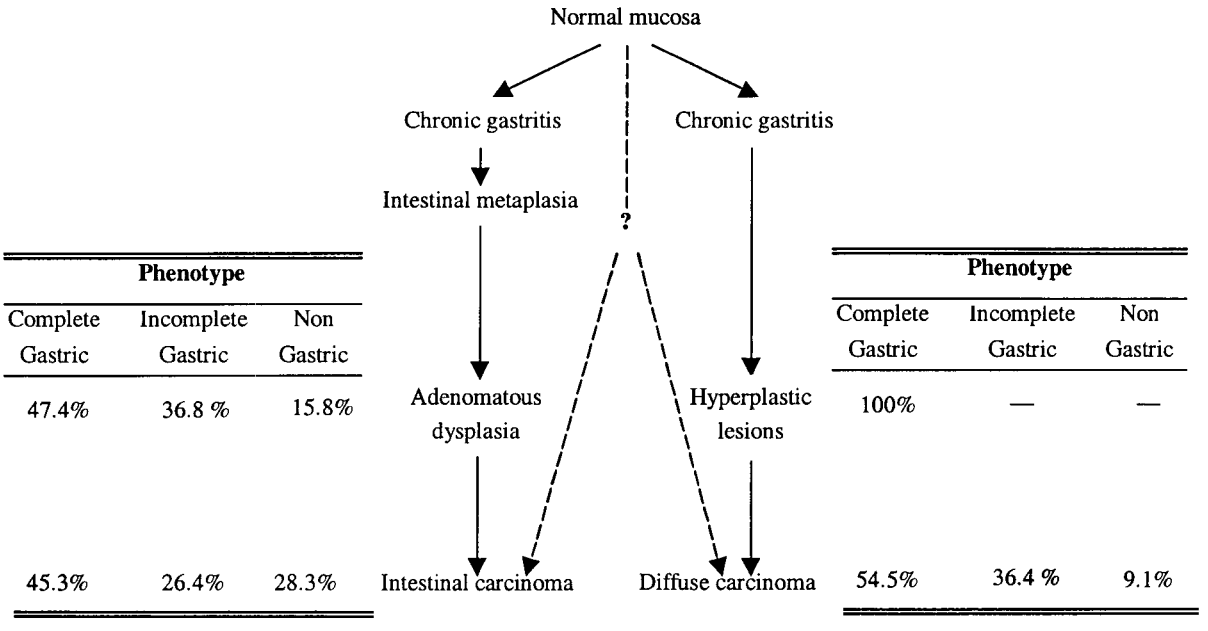
MUC1 is a very ubiquitous epithelial mucin[14,19] whereas MUC2 is characteristically expressed in goblet cells of native intestinal epithelium.[14,20] In the present study MUC1 expression was observed in the majority of the cases (82.3%) whereas MUC2 was expressed in relatively few cases (22.9%). We found no significant relationship between

the expression of both MUC1 and MUC2 and the histotype and cell differentiation pattern of the tumours, in keeping with previously reported data.[19-21] The expression of MUC2 in carcinomas displaying a gastric phenotype may indicate the existence of a mixed differentiation (gastric and intestinal) in these tumours; this possibility remains however disputable because we observed a low prevalence of MUC2 expression in the group of tumours that in principle should depict the highest degree of intestinal differentiation: carcinomas of the intestinal histotype with a non-gastric phenotype.

It is generally accepted that there are two main pathways of malignant transformation of gastric mucosa: one *via* intestinal metaplasia and adenomatous (flat or polypoid) dysplasia, leading to intestinal carcinomas; the other *via* hyperplastic or *de novo* changes, with or without concurrent non-metaplastic dysplasia, leading to diffuse carcinomas and to a subset of intestinal carcinomas[6,31] (Fig. 4).

In previous studies, we analysed the cell differentiation phenotypes of distinct pre-malignant lesions of the stomach, through the evaluation of the expression patterns of trefoil peptides and mucins.[17,18,22] We observed that complete gastric differentiation is a feature of hyperplastic lesions and that the cell differentiation of adenomatous lesions is heterogeneous, encompassing both gastric (complete and incomplete) and non-gastric phenotypes. In figure 4 we summarised the results obtained in the present study together with the aforementioned results having as background the model of gastric carcinogenesis previously mentioned. The comparison of these results highlights four points: firstly, the demonstration that intestinal metaplasia of incomplete type displays immunohistochemical markers of gastric differentiation, such as gastric type mucins[17] and TFF1[22] fits with the hypothesis that, in the cascade of events leading to intestinal carcinoma, incomplete intestinal metaplasia (namely type III) may serve as precursor of the adenomatous/dysplastic lesions exhibiting signs of gastric differentiation. Secondly, the three types of cell differentiation are similarly prevalent in the adenomatous lesions and in the intestinal type carcinomas (Fig. 4) thus supporting the hypothesis that adenomatous/dysplastic lesions do serve as precursors of intestinal-type gastric carcinoma. Thirdly, cell differentiation of gastric type is characteristically retained along the process of gastric carcinogenesis that leads to diffuse carcinomas (Fig. 4), in keeping with the origin of these tumours either *de novo* or from hyperplastic lesions.[32] Fourthly,

we observed that the absence (loss?) of signs of gastric differentiation occurs in both pathways of gastric carcinogenesis, though more prominently in that leading to intestinal-type carcinomas than that leading to diffuse carcinomas.



Phenotype				Phenotype		
Complete Gastric	Incomplete Gastric	Non Gastric		Complete Gastric	Incomplete Gastric	Non Gastric
47.4%	36.8 %	15.8%	Adenomatous dysplasia	100%	—	—
45.3%	26.4%	28.3%	Intestinal carcinoma	54.5%	36.4 %	9.1%
			Diffuse carcinoma			

Fig. 4: Simplified model of gastric carcinogenesis illustrating the two main pathways of malignant transformation, and the prevalences of complete gastric, incomplete gastric and non-gastric phenotypes observed in the different types of pre-malignant and malignant lesions. (Data on the premalignant lesions were previously published[18,22]).

Finally our results indicate that the absence (loss?) of gastric differentiation appears to be related with tumour progression, increasing from early to advanced carcinomas, both in intestinal and diffuse carcinomas (e.g. the only three diffuse carcinomas displaying a non-gastric phenotype were advanced cases). Ultrastructural and immunohistochemical data on record[1,5,7,8] led to the suggestion that there is acquisition of signs of intestinal differentiation along with gastric carcinoma progression. Our data support this assumption but did not attain the threshold of statistical significance. A detailed study of MUC2 and other putative markers of intestinal differentiation in a larger series of early and advanced carcinomas and, within the latter, in superficial and deep areas appears thus necessary to find whether or not the loss of gastric differentiation along with tumour progression is paralleled by intestinalisation of the carcinomas.

ACKNOWLEDGEMENTS

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ANNEX

Fig. 1

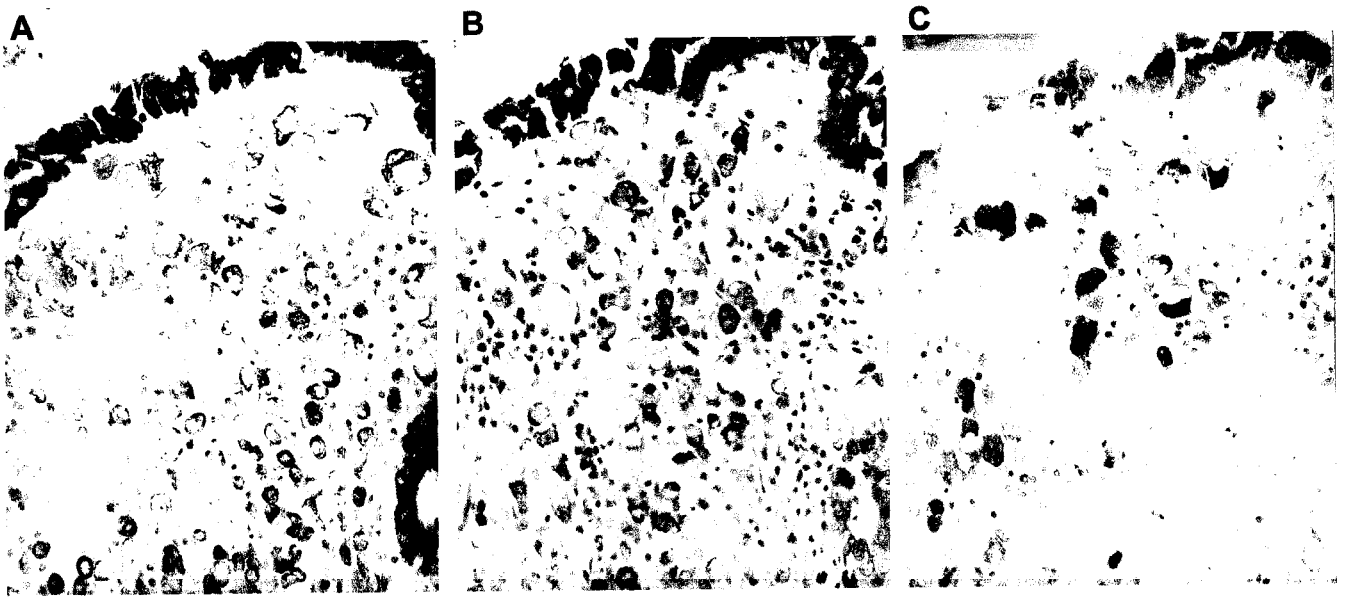


Fig. 2

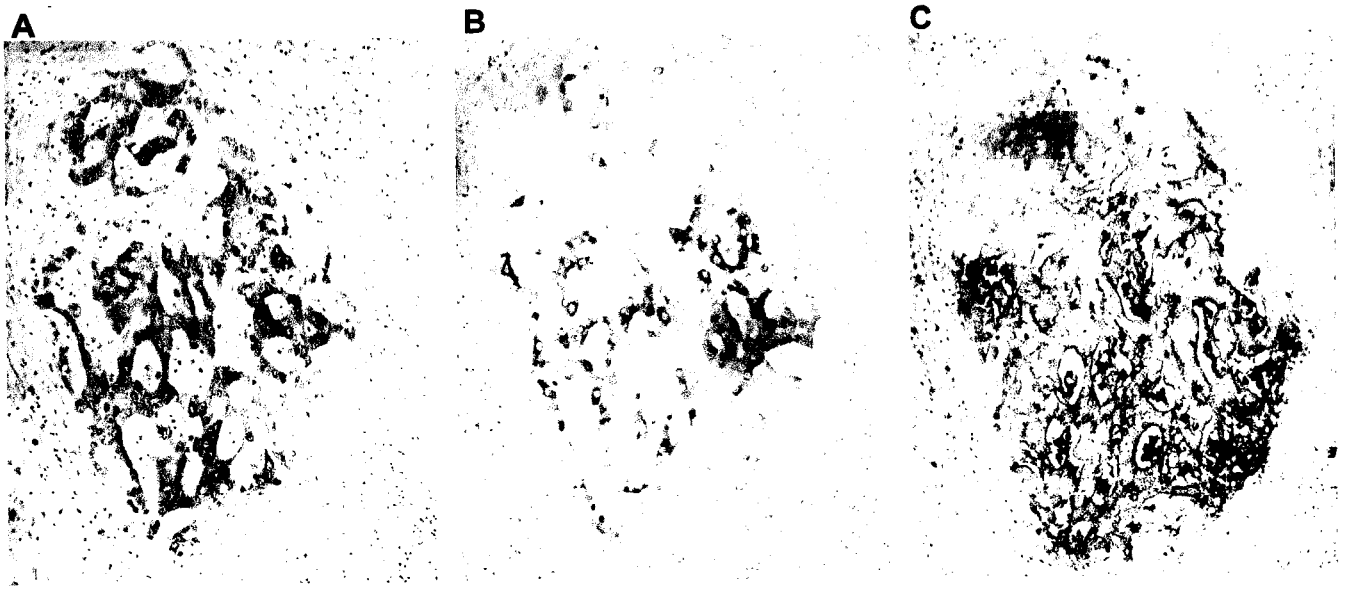
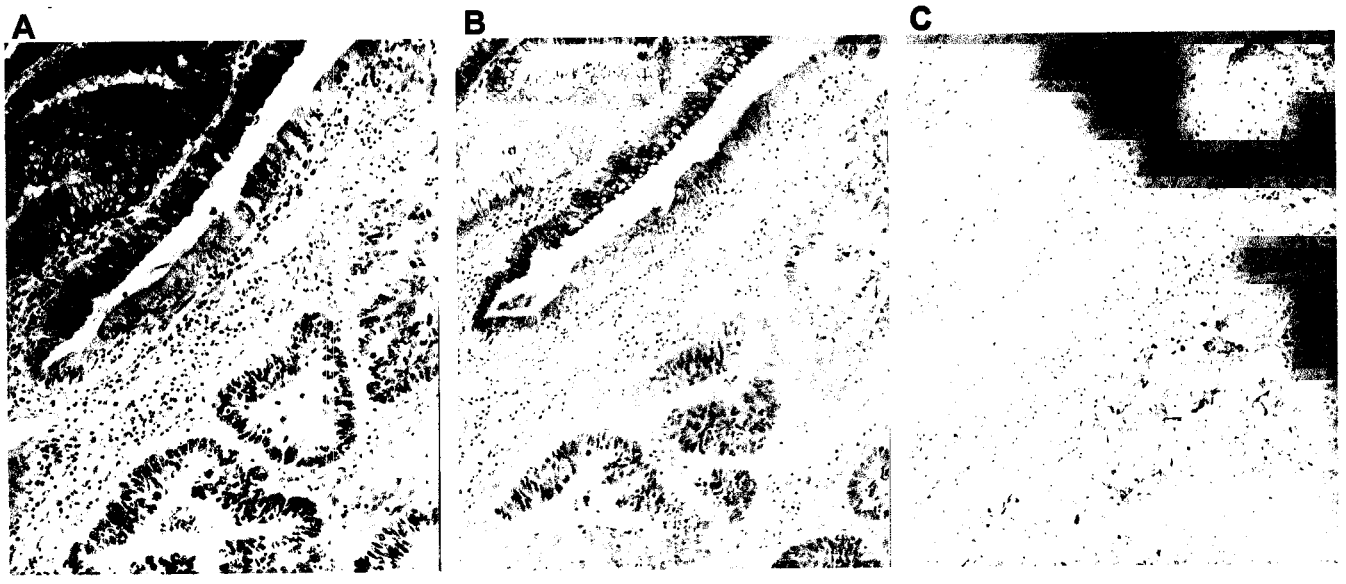


Fig. 3



PAPER V

International Journal of Surgical Pathology 6(3):135–144, 1998

E-Cadherin Expression Is Correlated with the Isolated Cell/Diffuse Histotype and with Features of Biological Aggressiveness of Gastric Carcinoma

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We studied the pattern of immunohistochemical expression of E-cadherin in a series of 50 gastric carcinomas, aiming to analyze its relationship with histotype and features of biological aggressiveness of the tumors and survival of the patients. Abnormal E-cadherin expression was significantly (p=.0007) higher in diffuse/isolated-cell-type carcinomas than in intestinal/glandular carcinomas. In mixed carcinomas abnormal E-cadherin expression in the diffuse/isolated-cell-type component (94.4%) was significantly (p=.007) higher than in intestinal/glandular component (55.6%). Significant relationships were observed between abnormal E-cadherin expression and nodal metastases (p=.004) and pTNM stages (p=.05). Survival of patients with tumors displaying abnormal E-cadherin expression was worse than that of patients with tumors presenting normal expression, though not attaining the threshold of statistical significance (p=.19). We conclude that abnormal E-cadherin expression is correlated with diffuse/isolated-cell histotype and features of biological aggressiveness of gastric carcinoma. *Int J Surg Pathol 6(3):135–144, 1998*
Key words: cancer, cell adhesion, E-cadherin, gastric carcinoma, prognosis, stomach.

E-cadherin belongs to the cadherin family of Ca^{2+} dependent cell adhesion molecules [1]. These molecules are localized at the adherens junction of epithelial cells and regulate the process of homophilic/homotypic adhesion between epithelial cells [1]. E-cadherin is a transmembrane glycoprotein presenting at its extracellular part (N-terminal) conserved tandem repeated sequences, known as cadherin domains [2]. Each of these domains contains two highly conserved Ca^{2+}-binding motifs [2], which play a key role in adhesion specificity [3]. The function of E-cadherin is also dependent on the interaction of its conserved cytoplasmic domain (C-terminal) with the cytoskeleton via molecular complexes involving α-, β- and γ-catenin [2]. The E-cadherin protein is expressed in all normal epithelial tissues: polarized at the lateral cell-cell boundaries in ductal and glandular epithelia and localized circumferentially in the whole cell membrane in squamous and transitional epithelia [4].

It has been postulated that changes in expression or function of E-cadherin may account for the ability of cancer cells to detach from the parent tumor structure and invade locally. Indeed, reduction

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and/or loss of expression of E-cadherin has been observed in several types of carcinoma, and frequently associated with poorly differentiated/undifferentiated carcinomas and/or invasive tumors [5–10]. Loss of expression of E-cadherin has been associated with mutation of the E-cadherin gene and loss of heterozygosity (LOH) in 16q22 in infiltrative lobular carcinomas of the breast [11,12]. Becker et al. [13] described mutations of the E-cadherin gene in 50% of diffuse carcinomas of the stomach and reported a gastric carcinoma case presenting LOH with retention of a mutated E-cadherin allele. These findings support a role of E-cadherin as a tumor and/or invasion suppressor gene, as previously suggested by in vitro observations [14,15].

The first descriptions of E-cadherin expression in gastric carcinoma were provided by Shimoyama and Hirohashi [16] and Shiozaki et al. [17]. While Shiozaki et al. [17] reported a reduced E-cadherin expression in poorly differentiated/undifferentiated carcinomas, Shimoyama and Hirohashi [16] could not find any correlation between E-cadherin expression and the differentiation of the tumors. This initial discrepancy was clarified by further studies on the expression of E-cadherin in gastric carcinoma that pointed to a trend toward an association between reduction/loss or abnormal expression of E-cadherin and two histotypes of gastric carcinoma: poorly differentiated/undifferentiated carcinomas and diffuse carcinomas [5,18–23]. Moreover, in some of these studies a significant relationship was observed between altered expression of E-cadherin and features of aggressiveness of the tumors and/or poor survival of the patients [5,18,19,22,23].

Altogether, the data on record support the existence of a close association between altered expression of E-cadherin and infiltrative/invasive growth pattern of the tumors with reduced or no adhesion between cancer cells, features that are shared by both diffuse gastric carcinoma [19,23] and invasive lobular carcinoma of the breast [10].

We undertook the present immunohistochemical study of E-cadherin expression in a series of gastric carcinomas in an attempt to settle the aforementioned controversy on the putative association between altered expression of E-cadherin and the different histotypes of gastric carcinoma. The study of E-cadherin expression was carried on in carcinomas of "pure" histologic type (intestinal/glandular carcinomas and diffuse/isolated-cell-type carcinomas) as well as in a subgroup of mixed gastric carcinomas [24]. In these mixed carcinomas E-cadherin expression was evaluated separately in the different histologic components of the tumors. To the best of our knowledge this is the first report describing differences in E-cadherin expression in the different histologic components of mixed gastric carcinomas.

Materials and Methods

Tissue Material

Surgical specimens from 50 gastric carcinomas consecutively resected at Hospital S.João-Medical Faculty, Porto, Portugal, were studied. The noninvolved mucosa adjacent to each case was also studied.

The tissue fragments were fixed in 10% formaldehyde and embedded in paraffin. Serial sections of 4 μm were cut from each block and used for routine staining with hematoxylin and eosin (H&E) and immunohistochemical study.

Immunohistochemistry

Monoclonal antibody (MoAb) HECD-1 (Zymed, San Francisco, CA) was used for immunohistochemical (IHC) study of the expression of E-cadherin in formalin-fixed paraffin-embedded tissues. A modification of the avidin-biotin-peroxidase complex method [25] was applied with an additional step for microwave antigen retrieval. The paraffin sections (4 μm thick) were dewaxed and antigen retrieval was done by microwave treatment 5 × 1.5 min at 750 W in a 0.1% detergent solution [26]. The sections were treated with 0.3% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase, washed in TBS, and then incubated for 20 minutes with normal rabbit serum, at a dilution of 1:5 in TBS containing 25% bovine serum albumin (BSA). Excess normal serum was removed and replaced by the MoAb HECD-1 diluted at 1:400. After overnight incubation (18 hours) at 4°C, slides were washed with TBS and the sections incubated with a 1:200 dilution of biotin-labeled secondary antibody for 30 minutes. After washing with TBS, sections were incubated with avidin-biotin-peroxidase complex (10 mg/mL of biotin-labeled peroxidase) for 60 minutes. This was followed by staining the sections for 6 minutes with 0.05% diaminobenzidine, freshly prepared in 0.05 M TRIS buffer, pH 7.6, containing 0.01% hydrogen peroxide. Finally, sections were counterstained with hematoxylin, dehydrated, and mounted. Dilution of primary antibodies, biotin-labeled secondary antibodies, and avidin-biotin-peroxidase complex were made with TBS containing 2.5% BSA.

All series included normal gastric mucosa as positive controls. Negative controls were performed by substitution of the primary antibody with IgG1 immunoglobulins of the same subclass and concentration as the MoAb.

Scoring

For purposes of data analysis, all tumors with loss of normal membranous pattern of staining were classified as abnormal. Staining was scored in a semiquantitative fashion from 0 to 3, according to the evaluation criteria described by Jawhari et al. [5]: 0, absence of staining; 1, diffuse cytoplasmic staining; 2, heterogeneous staining (i.e., both normal and abnormally staining areas); and 3, normal membranous pattern of staining. Whenever the staining patterns varied within the same tumor, the score was based on the dominant pattern, provided this was observed in more than 90% of neoplastic cells. Cases exhibiting more than one pattern of immunoreactivity were classified as heterogeneous whenever each of the patterns was observed in more than 10% of neoplastic cells.

Statistical Analysis

The statistical analysis of the results was performed by Pearson-χ^2 test. Differences were considered to be statistically significant at values of $p<.05$.

The relationship between the expression of E-cadherin and survival of the patients was assessed by use of both univariate and multivariate analysis. Survival curves were calculated according to the method of Kaplan and Meier and compared statistically by the Generalized Savage (Mantel-Cox) test. The Cox's proportional hazards model was employed to evaluate the influence on survival of E-cadherin expression in relation to other clinicopathologic variables. The MPLR (maximum partial likelihood ratio) method was employed to perform a linear stepwise multivariate regression of the factors that are significantly or suggestively related to the survival. The assumed limit for significance to enter a term was .1 and the limit to remove a term .15.

Results

Normal Mucosa

E-cadherin staining was observed in every case throughout the gastric epithelium of noncancerous areas, including areas of intestinal metaplasia. At the cellular level, E-cadherin expression was mainly localized at the lateral cell-cell boundaries, but staining was also seen in the paranuclear Golgi area (Fig. 1).

Gastric Carcinoma

Table 1 summarizes the clinicopathologic features of the 50 cases included in the present series and the immunohistochemical findings regarding E-cadherin expression.

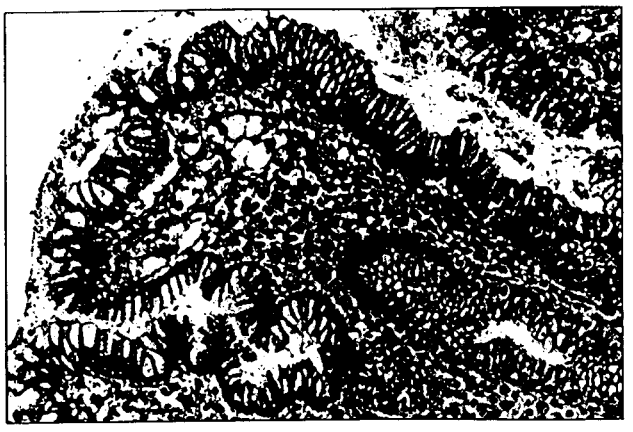

Fig. 1. Normal gastric mucosa: E-cadherin immunoreactivity is observed at the lateral cell-cell boundaries throughout the gastric epithelium.

According to Laurén's classification [27], cases were classified as intestinal (n=20), diffuse (n=28), and unclassifiable (n=two). According to the classification proposed by Carneiro et al. [24], cases were classified as glandular (n=19), isolated-cell-type (n=11), solid (n=two), and mixed (n=18).

A normal pattern of E-cadherin expression, i.e., a membranous staining pattern, was observed in 13 of the 50 carcinomas (26.0%) of the whole series. This pattern of immunoreactivity was observed in 11/20 (55.0%) intestinal carcinomas (Fig. 2A) and in 2/28 (7.1%) diffuse carcinomas (Fig. 2B). Intestinal carcinomas displayed immunoreactivity at the lateral cellular membrane (polarized pattern) (Fig. 2A), whereas, in diffuse carcinomas, membranous staining was localized in the whole cell membrane (nonpolarized pattern) (Fig. 2B).

Abnormal pattern of E-cadherin staining was observed in 37 of the 50 gastric carcinomas (74.0%). In intestinal carcinomas we observed an abnormal pattern of E-cadherin expression in 9/20 (45.0%) cases (Fig. 3A), encompassing cases scored as: 1, 3/9 (33.3%); and 2, 6/9 (66.7%). Abnormal pattern of E-cadherin expression was observed in 26/28 (92.9%) diffuse carcinomas (Fig. 3B), encompassing cases scored as: 0, 1/26 (3.8%); 1, 11/26 (42.3%); and 2, 14/26 (53.8%). The comparison of the results obtained in the two histotypes yielded statistically significant differences (p=.0007) (Table 1).

Eighteen cases, classified according to the predominant histologic type as intestinal or diffuse carcinomas, displayed small foci with a structure different from the main component of the tumor—foci of intestinal carcinoma in diffuse carcinomas (n=17) and foci of diffuse carcinoma in an intestinal carcinoma (n=1). These cases were subclassified, according to the classification proposed by Carneiro et al. [24], as mixed carcinomas. In these cases, immuno-

Table 1. Relationship Between the Clinicopathologic Features of Gastric Carcinomas and E-cadherin Expression

		E-cadherin IHC		
	No. of Cases (%)	Normal (%)	Abnormal (%)	p Value
Age				
<40 years	7 (14.0)	1 (14.3)	6 (85.7)	NS
40–65 years	27 (54.0)	8 (29.6)	19 (70.4)	
≥65 years	16 (32.0)	4 (25.0)	12 (75.0)	
Gender				
Male	34 (68.0)	10 (29.4)	24 (70.6)	NS
Female	16 (32.0)	3 (18.8)	13 (81.3)	
Laurén's classification				
Intestinal	20 (40.0)	11 (55.0)	9 (45.0)	.0007
Diffuse	28 (56.0)	2 (7.1)	26 (92.9)	
Unclassified	2 (4.0)	0 (0.0)	2 (100.0)	
Carneiro, et al. classification				
Isolated-cell ca	11 (22.0)	1 (9.1)	10 (90.9)	
Glandular ca	19 (38.0)	11 (57.9)	8 (42.1)	.001
Solid ca	2 (4.0)	0 (0.0)	2 (100.0)	
Mixed ca	18 (36.0)	1 (5.6)	17 (94.4)	
Depth of invasion				
T1 (mucosa + submucosa)	5 (10.0)	1 (20.0)	4 (80.0)	
T2 (muscular + subserosa)	7 (14.0)	3 (42.9)	4 (57.1)	NS
≥T3 (serosa + adjacent organs)	38 (76.0)	9 (23.7)	29 (76.3)	
Venous invasion				
Absent	30 (60.0)	8 (26.7)	22 (73.3)	NS
Present	20 (40.0)	5 (25.0)	15 (75.0)	
Lymph node metastasis				
Absent	18 (36.0)	9 (50.0)	9 (50.0)	.004
Present	32 (64.0)	4 (12.5)	28 (87.5)	
pTNM stage				
I	16 (32.0)	8 (50.0)	8 (50.0)	
II	8 (16.0)	2 (25.0)	6 (75.0)	.05
III	25 (50.0)	3 (12.0)	22 (88.0)	
IV	1 (2.0)	0 (0.0)	1 (100.0)	
Survival				
<3 years	35 (70.0)	5 (14.3)	30 (85.7)	.004
≥3 years	15 (30.0)	8 (53.3)	7 (46.7)	
Total	50 (100.0)	13 (26.0)	37 (74.0)	

NS: not significant.

histochemical expression of E-cadherin was evaluated separately in each component of the tumor (Table 2). Seventeen cases displayed an overall abnormal pattern of E-cadherin expression corresponding, according to Laurén's classification, to 16 diffuse carcinomas and to one intestinal carcinoma. The remaining case exhibited a normal pattern of immunoreactivity.

In seven of these mixed carcinomas a normal pattern of immunoreactivity was observed in the intestinal/glandular component and an abnormal pattern was seen in the diffuse/isolated-cell component (Table 2) (Fig. 4). In 10 cases both components of mixed carcinomas displayed an abnormal pattern of immunoreactivity and, in the remaining case, a normal pattern of immunostaining was observed in the two components (Table 2).

In the whole series, we found a significant association between E-cadherin staining and metastization to lymph nodes (p=.004) and pTNM staging (p=.05) (Table 1). No significant relationship was found between E-cadherin expression and the age or gender of patients, depth of penetration of gastric wall, and venous invasion (Table 1).

Regarding the survival time, the patients were divided into two groups: short survivors (survival time <3 years) and long survivors (survival time ≥3 years). The percentage of cases with abnormal E-cadherin expression was significantly higher in short survivors (85.7%) than in long survivors (46.7%) (p=.004) (Table 1). In univariate analysis of survival, the survival time of patients with gastric carcinomas displaying normal E-cadherin expression (mean survival time, 42.3 months) was better than that of patients with an abnormal staining pattern (mean survival time, 29.1 months), though the comparison of the respective survival curves did not reach the threshold of statistical significance (p=.19) (Fig. 5). The multivariate analysis of the whole series showed that venous invasion was the strongest independent prognostic factor followed by pTNM stage. E-cadherin expression did not stand as an independent prognostic factor.

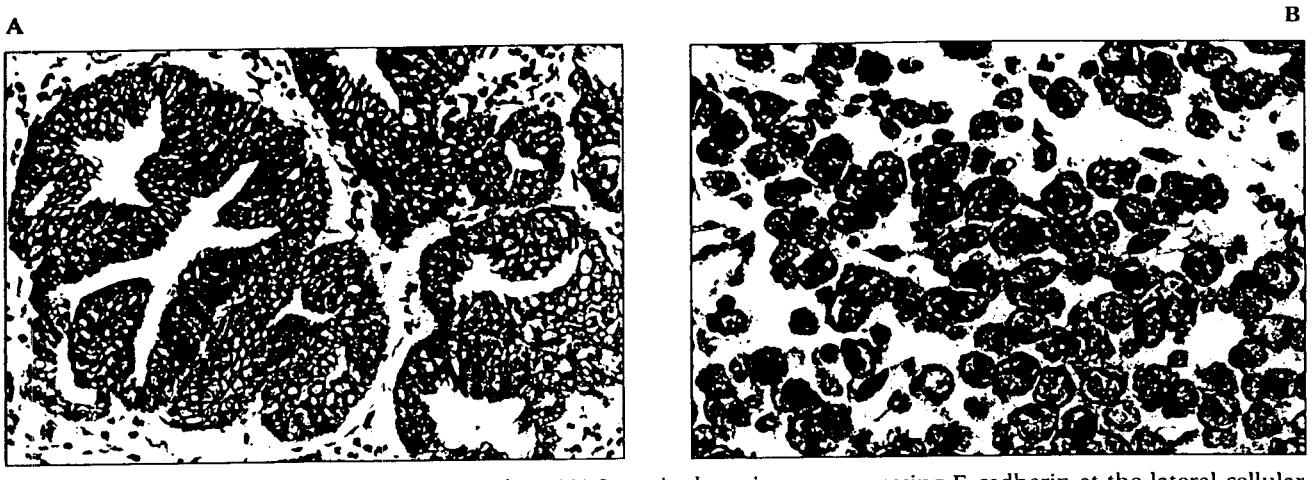

Fig. 2. Normal pattern of E-cadherin expression: (A) Intestinal carcinoma expressing E-cadherin at the lateral cellular membrane (polarized pattern); (B) Diffuse carcinoma showing membranous staining localized in the whole cell membrane (nonpolarized pattern).

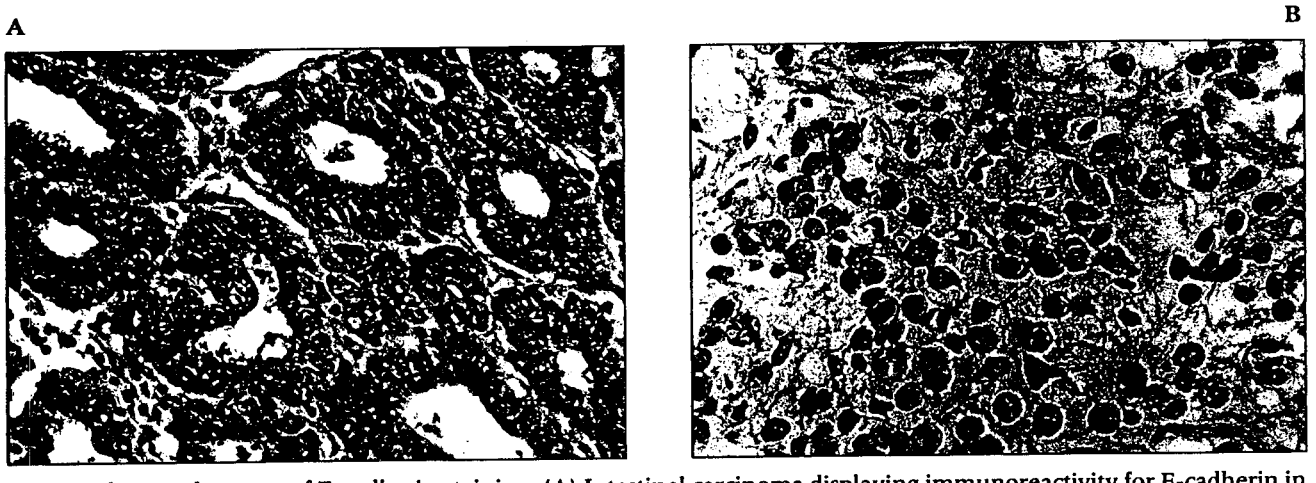

Fig. 3. Abnormal pattern of E-cadherin staining: (A) Intestinal carcinoma displaying immunoreactivity for E-cadherin in the cytoplasmic and paranuclear Golgi area; (B) Diffuse carcinoma with E-cadherin faintly expressed in the cytoplasm of the neoplastic cells.

Discussion

We found abnormal E-cadherin expression in 74.0% of gastric carcinomas, a prevalence close to that reported by Yonemura et al. [19] and Gabbert et al. [22]. In other studies [5,17,18,20,21], the percentage of altered E-cadherin expression in gastric carcinoma ranged from about 17% in the study of Shimoyama and Hirohashi [16] to about 92% in the study of Mayer et al. [23]. Differences in the antibodies and in the evaluation criteria used in the different series probably account for the aforementioned discrepancies.

According to the system we used to score immunoreactivity in our series, one case was scored as 0 (absence of immunoreactivity), 16 cases were scored as 1 (diffuse cytoplasmic staining), and 20 cases were scored as 2 (heterogeneous staining). Altogether, these results show that, in a large proportion of cases (36/50), E-cadherin immunoreactivity was detected in the cytoplasm (alone or in combination with expression at the cell membrane). Cytoplasmic localization of E-cadherin in gastric carcinoma had already been described by other authors [5,17,22]. Several processes may account for this ectopic localization of E-cadherin: endocytic inter-

Table 2. Pattern of E-cadherin Immunoexpression in Separate Histologic Components of 18 Cases of Mixed Gastric Carcinoma

Case	Predominant Component	Pattern of IHC (Score)	
		Diffuse/Isolated-Cell-Type Component	Intestinal/Glandular Component
1	Diffuse	Abnormal (0)	Abnormal (0)
2	Diffuse	Abnormal (1)	Abnormal (1)
3	Diffuse	Abnormal (1)	Abnormal (2)
4	Diffuse	Abnormal (1)	Abnormal (2)
5	Diffuse	Abnormal (1)	Abnormal (2)
6	Diffuse	Abnormal (1)	Abnormal (0)
7	Diffuse	Abnormal (1)	Abnormal (2)
8	Diffuse	Abnormal (1)	Abnormal (2)
9	Diffuse	Abnormal (1)	Abnormal (2)
10	Intestinal	Abnormal (2)	Abnormal (2)
11	Diffuse	Abnormal (1)	Normal (3)
12	Diffuse	Abnormal (1)	Normal (3)
13	Diffuse	Abnormal (1)	Normal (3)
14	Diffuse	Abnormal (1)	Normal (3)
15	Diffuse	Abnormal (1)	Normal (3)
16	Diffuse	Abnormal (1)	Normal (3)
17	Diffuse	Abnormal (1)	Normal (3)
18	Diffuse	Normal (3)	Normal (3)

0, absence of staining; 1, diffuse cytoplasmic staining; 2, heterogeneous staining (i.e., both normal and abnormally staining areas); 3, normal membranous pattern of staining.

nalization of cell contact domains containing junctional cadherins [28]; deficient transport of an abnormal E-cadherin protein (possibly due to the occurrence of mutations in the E-cadherin gene) into the endoplasmic reticulum (ER); retrograde transport of the protein from the ER to the cytoplasm involving the detection of protein abnormalities by the quality control system present in the ER [29]; and, finally, we cannot exclude the possibility that the diffuse cytoplasmic localization of E-cadherin may be due to the vesiculation of the Golgi apparatus, a structural change that has been described to occur in tumor cells [30].

We found a significantly higher frequency of abnormal E-cadherin expression in diffuse carcinomas (92.9%) than in intestinal carcinomas (45.0%). This finding is in agreement with those reported by other authors who also found a correlation between altered expression of E-cadherin and either diffuse or poorly differentiated/undifferentiated gastric carcinoma [5,17–23]. In our series, the correlation between histotype and E-cadherin expression was maintained when the cases were classified according to the classification proposed by Carneiro et al. [24] into glandular, isolated-cell-type, solid, and mixed carcinomas. The percentage of cases exhibiting abnormal expression of E-cadherin was significantly higher in isolated-cell-type (90.9%) and mixed (94.4%) carcinomas than in glandular carcinomas (42.1%). The high prevalence of mixed carcinomas displaying abnormal E-cadherin expression (94.4%) is in agreement with the fact that in 17/18 mixed carcinomas the predominant component was constituted by diffuse/isolated-cell-type carcinoma. The separate evaluation of E-cadherin expression in distinct histologic components of the mixed carcinomas consistently reproduced the association between E-cadherin expression pattern and the histotype. E-cadherin expression was scored as

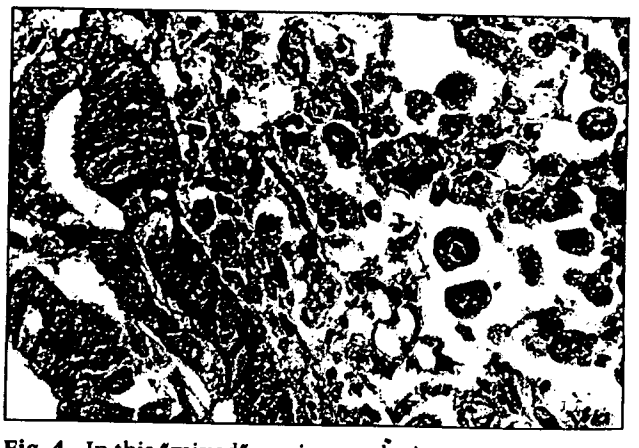

Fig. 4. In this "mixed" carcinoma the immunoexpression pattern of E-cadherin differs in each component of the tumor: Intestinal component (left) displays a normal pattern of immunoreactivity (polarized at the lateral cell membrane), whereas the diffuse component (right) depicts abnormally expressed E-cadherin (diffuse in the cytoplasm).

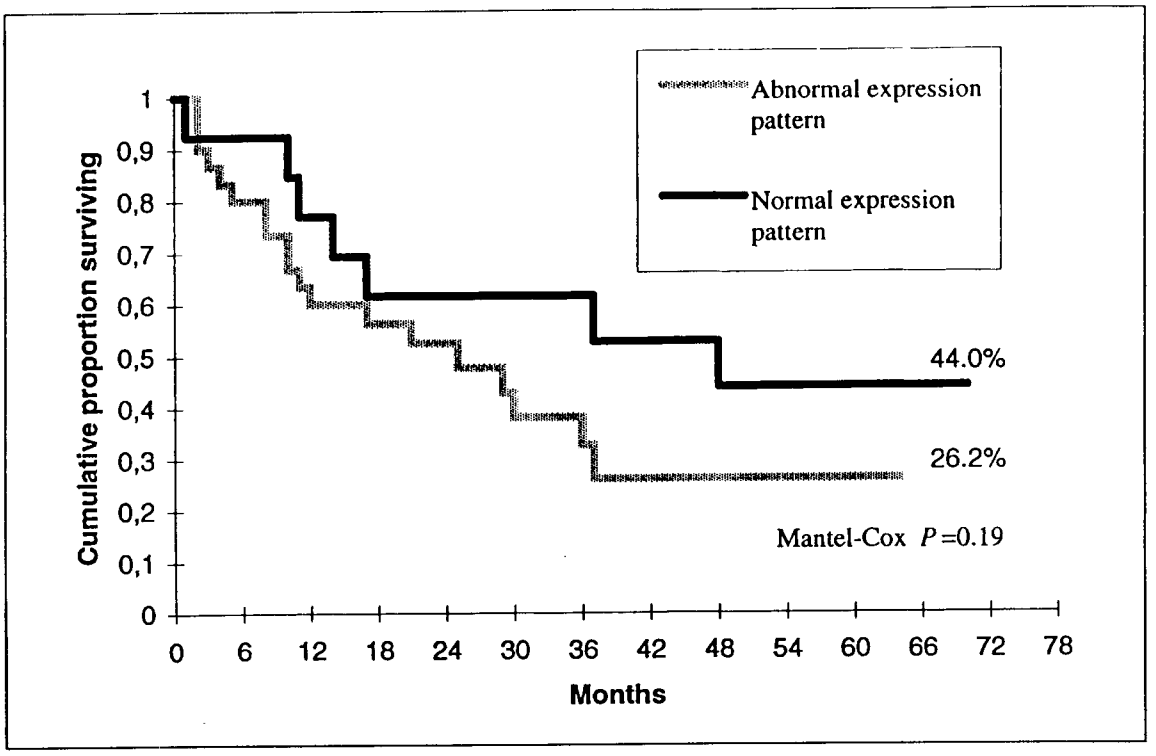

Fig. 5. Survival curves of patients according to the pattern of E-cadherin expression in gastric carcinomas.

abnormal in the diffuse/isolated-cell-type component in 17/18 (94.4%) mixed carcinomas. At variance with this, in the intestinal/glandular component of mixed carcinomas, abnormal E-cadherin expression was observed in 10/18 (55.6%) cases, the remaining eight cases (44.4%) exhibiting, in the glandular areas, a polarized pattern of E-cadherin expression at the cell membrane.

The significant relationship between an abnormal expression of E-cadherin and diffuse/isolated-cell-type carcinomas, which is kept both in "pure" carcinomas and in the diffuse component of mixed carcinomas, is in keeping with the hypothesis that E-cadherin plays a crucial role in the adhesion among neoplastic cells, which, if disrupted, leads to dissociation of cells and scattered growth. This hypothesis is challenged by the existence of a small percentage of diffuse/isolated-cell-type carcinomas displaying a normal E-cadherin expression and by the occurrence of carcinomas with glandular structure, in which neoplastic cells are cohesive, despite an abnormal expression of E-cadherin. In the latter group, one cannot exclude that E-cadherin is still expressed at the junctional complexes of the cell membrane at a level not easily detectable by immunohistochemistry. Furthermore, it is admissible, on the basis of evidence previously reported [31], that the abnormal expression of E-cadherin may reflect a temporary modulation phenomenon occurring in tumors that are heterogeneous regarding E-cadherin expression, i.e., displaying E-cadherin both in the cytoplasm and at the cell membrane. In fact, recent studies suggest that modulation of E-cadherin expression in carcinomas is a very complex process, subjected to temporary microenvironmental influences as demonstrated by Mareel et al. [31]. These authors showed that injection into nude mice of homogeneously E-cadherin-positive cells, which were noninvasive *in vitro*, led to the formation of invasive and sometimes metastatic tumors that were heterogeneous with regard to E-cadherin expression. According to these data and as put forward also by other authors [32–34], loss of E-cadherin could be, at least in some tumors, a temporary phenomenon that would allow invasion to last long enough for metastization to occur. In keeping with this hypothesis, the majority of tumors with intestinal/glandular phenotype from our series, with ab-

normal expression of E-cadherin, correspond to cases showing a heterogeneous pattern of E-cadherin expression (score 2), characterized by the coexistence of areas displaying normal (membranous) E-cadherin expression and areas exhibiting abnormal (cytoplasmic) expression of E-cadherin.

Regarding the isolated-cell-type/diffuse carcinomas ("pure" histotypes or components of mixed carcinomas) displaying a membranous expression of E-cadherin, one has to admit that in such cases the adhesion of neoplastic cells is disrupted by an alteration at another level of the cell adhesion system. In fact, E-cadherin is only part of a complex cell adhesion system and its function is dependent on the interaction of its conserved cytoplasmic domain (C-terminal) with the cytoskeleton via molecular complexes involving α-, β-, and γ-catenin. Alterations in the associated cytoplasmic molecules, such as the catenins, are thought to impair cell adhesion in tumors exhibiting normal E-cadherin expression. This hypothesis is supported by *in vitro* experimental evidence [35] and by data on record on the expression of catenins in several types of cancer [5,8,36–39]. Jawhari et al. [5] studied the expression of E-cadherin and α-, β-, and γ-catenin in a series of 89 gastric carcinomas and reported abnormal expression, of at least one of the antigens, in about 90% of the cases. Moreover, Jawhari et al. [5] found a strong correlation between abnormal expression of the E-cadherin/catenin complex and the diffuse type of gastric carcinoma. Furthermore, recent data indicate that phosphorylation of tyrosine residues of β-catenin, via epidermal growth factor receptor and/or c-erbB2 activation, may inhibit the assembly of the E-cadherin-catenin-cytoskeleton complex, leading to the disruption of the adhesion system [40]. These mechanisms would help to explain the group of tumors with diffuse/isolated-cell phenotype that keep normal membranous expression of E-cadherin and, nevertheless, display a scattered structure with complete lack of adhesiveness between the tumor cells.

Remaining to be fully elucidated is the role played by mutations in E-cadherin gene regarding the abnormal pattern of E-cadherin expression as observed at the immunohistochemical level. In a series of 53 gastric carcinomas studied by Becker et al. [13], E-cadherin mutations were detected in 13/26 (50.0%) diffuse carcinomas. Our own preliminary results on the study of alterations in the E-cadherin gene point to a similar frequency of E-cadherin gene mutations in diffuse/isolated-cell-type gastric carcinomas (unpublished data). Similarly, mutations of the E-cadherin gene have also been described in a significant proportion of infiltrative lobular breast carcinomas, which also display a "diffuse" phenotype with an infiltrative growth pattern and scattered tumor cells [11,12]. Altogether, these results provide a genetic basis for the existence of (permanent) alterations of E-cadherin expression/function in diffuse/isolated-cell-type carcinomas of the stomach.

Besides the role of E-cadherin in the establishment of the tumor histotype, it has also been suggested that alterations in E-cadherin expression may contribute to explain the invasive and metastatic abilities of the tumors. In our series we found no relationship between E-cadherin expression and local invasion, in keeping with the fact that invasion is a complex process also involving mechanisms other than disruption of cell-cell adhesion, such as alterations of cell-matrix interactions and extracellular protease activity [41]. No significant relationship was also observed between E-cadherin expression and venous invasion, suggesting that the ability of neoplastic cells to invade vessels is not entirely dependent on the ability to dissociate from each other. Furthermore, Cowley et al. [42] showed that in 40% of adenocarcinomas E-cadherin levels are elevated in their intravascular tumor compartment in comparison to their extravascular compartments, pointing to the possibility of modulation of E-cadherin expression.

We observed a significant association between abnormal E-cadherin expression and the presence of lymph node metastases and high pTNM stages. Our results are in agreement with those reported by other authors [18–20,23,36], although such relationships could not be demonstrated by Gabbert et al [22] in a series of 413 gastric carcinomas. These apparent discrepancies are probably influenced by the different criteria used in the evaluation of the immunoreactivity. The relationship between abnormal E-cadherin expression and the aforementioned parameters of biological aggressiveness is reflected on the survival of the patients with tumors displaying an abnormal pattern of E-cadherin expression. Our finding of the worse prognosis of patients with tumors displaying abnormal expression of E-cadherin is in accordance with those reported by Mayer et al. [23] and Gabbert et al. [22], who observed that patients with tumors presenting loss or abnormal expression of E-cadherin had statistically significant worse survival rate than those with tumors displaying normal E-cadherin expression.

In conclusion, our results show that abnormal expression of E-cadherin is strongly correlated with diffuse/isolated-cell histotype of gastric carcinoma. This feature is kept both in tumors with "pure" histologic type and in the diffuse/isolated-cell-type

component of mixed carcinomas, regardless of the pattern of E-cadherin expression in the other(s) component(s) of the mixed tumors. Further, abnormal E-cadherin expression is correlated with features of aggressiveness of the tumors and poor survival of the patients.

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PAPER VI

E-Cadherin Gene Mutations Provide a Genetic Basis for the Phenotypic Divergence of Mixed Gastric Carcinomas

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SUMMARY: Inactivation of the E-cadherin gene has been described previously in gastric carcinomas. In the present study, we investigated the alterations of the E-cadherin gene in gastric carcinomas and analyzed the relationship between such alterations and the histotypes of the tumors. We performed PCR/single-strain conformation polymorphism mutation screening and loss of heterozygosity analysis of the E-cadherin gene in a series of 26 gastric carcinomas, including 10 "pure" intestinal, 10 "pure" diffuse, and 6 mixed gastric carcinomas, the latter with intestinal and diffuse components. Fifteen mutations of the E-cadherin gene were identified in 12 cases (46.2%). Mutations included 10 missense mutations, 7 of which occurred in sequences coding for calcium binding motifs, 3 splice site mutations, 1 nonsense mutation, and 1 frameshift deletion. We found mutations of the E-cadherin gene in 7 of 10 "pure" diffuse carcinomas (70.0%) and in 5 of 6 mixed carcinomas (83.3%). No mutations were found in "pure" intestinal carcinomas. In mixed carcinomas, inactivating E-cadherin mutations were exclusively observed in the diffuse component of the tumors. We conclude that E-cadherin inactivation is significantly related with the diffuse histotype in gastric carcinomas, not only in "pure" diffuse carcinomas but also in the diffuse component of mixed tumors. To the best of our knowledge, this is the first report advancing a genetic basis for the phenotypic divergence of mixed gastric carcinomas. (*Lab Invest 1999, 79:1–000*).

E-Cadherin is a member of the cadherin family of calcium-dependent cell adhesion molecules (Takeichi, 1991). These molecules are localized in lateral cell-cell contacts of epithelial cells and regulate the process of homophilic/homotypic adhesion between epithelial cells (Takeichi, 1991). It has been postulated that changes in the expression or function of E-cadherin may account for the ability of cancer cells to detach from the parent tumor structure and invade locally. Indeed, abnormal E-cadherin expression (characterized by loss, reduced, and/or cytoplasmic expression) has been observed in several types of carcinoma, and is frequently associated with poorly differentiated/undifferentiated carcinomas and/or invasive tumors (Smith and Pignatelli, 1997). Moreover, mutations of the E-cadherin gene and/or loss of heterozygosity (LOH) in 16q22.1 have been reported in diffuse gastric carcinoma (Becker et al, 1994; Guilford et al, 1998; Muta et al, 1996), lobular carcinoma of the breast (Berx et al, 1995, 1996), and carcinomas of the endometrium and ovary (Risinger et al, 1994), supporting a role for E-cadherin as a tumor suppressor gene.

In gastric carcinoma, there is an association between abnormal expression of E-cadherin and the diffuse (isolated-cell) histotype (Gabbert et al, 1996; Jawhari et al, 1997; Machado et al, 1998; Mayer et al, 1993; Shino et al, 1995). Becker et al (1994) described mutations of the E-cadherin gene in 50% of diffuse carcinomas of the stomach. Recently, the report of germline mutations in kindred with early onset diffuse gastric carcinoma became the first description of a molecular basis for familial gastric cancer of the diffuse type (Gayther et al, 1998; Guilford et al, 1998).

In a previous study, we confirmed that abnormal E-cadherin expression was significantly associated with the diffuse type of gastric carcinoma (Machado et al, 1998) and we observed that in most mixed carcinomas the diffuse component exhibited an abnormal E-cadherin expression, whereas the intestinal component displayed a normal (polarized at the lateral cell membrane) pattern of E-cadherin expression (Machado et al, 1998). It remains to be elucidated whether the differential expression of E-cadherin, in individual histologic components of mixed carcinomas, represents an epigenetic phenomenon or, in

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alternative, reflects the existence of genotypically divergent clones.

In the current study, we performed PCR/single-strain conformation polymorphism (SSCP) mutation screening and LOH analysis of the E-cadherin gene in a series of gastric carcinomas. This study was carried on in carcinomas of "pure" histologic type as well as in a subgroup of mixed gastric carcinomas. In the latter group, molecular analysis was performed separately in the different histologic components of the tumors. Our main goal was to investigate whether or not the putative alterations of E-cadherin were restricted to the diffuse component of the mixed gastric carcinomas.

Results

Mutational Analysis of the E-Cadherin Gene

By using PCR/SSCP, we identified mobility shifts in 24 of the 26 gastric carcinoma cases. Comparison with SSCP patterns observed in normal DNA counterparts revealed that in 14 cases there were band shifts that were not tumor restricted, suggesting a polymorphic nature. Sequencing analysis of these bands revealed either silent or intronic substitutions, previously described as polymorphisms (Berx et al, 1998) (Table 1).

In 12 of the 26 cases (46.2%), we observed tumor restricted mobility shifts: 9 cases harbored a single shift, and 3 cases presented two different mobility shifts each (Table 2). In two of these cases there were also polymorphisms.

The sequencing of the tumor restricted bands resulted in the identification of 15 mutations, spanning the region between exons 7 and 12 of the E-cadherin gene (Table 2). One nonsense mutation and one frameshift deletion, resulting in premature downstream stop codons, were detected in cases 2 and 5, respectively. A G→A transition in the last nucleotide of intron 7, resulting in the loss of the normal acceptor splice site, was identified in three cases (cases 7, 13, and 16). Ten missense mutations were found, causing single amino acid substitutions: 7 missense mutations occurred in highly conserved sequences coding for calcium binding motifs of the E-cadherin extracellular domain (cases 3, 4, 5, 10, 11, 14, and 15); the remaining 3 missense mutations (cases 6, 7, and 16) took place in nonconserved sequences of the E-cadherin gene.

Table 1. Polymorphisms[a] Identified in the E-Cadherin Gene of Nine Patients with Gastric Carcinoma

Intron/Exon No.	Codon No.	Nucleotide change[b] (Aminoacid)	Frequency
Intron 4	—	531 + 10G→C	1/26
Exon 12	632	1896C→T (H→H)	1/26
Intron 12	—	1937-13T→C	3/26
Exon 13	692	2076C→T (A→A)	11/26
Exon 16	879	2637C→T (G→G)	2/26

[a] All these polymorphisms have been described previously (For review see Berx et al, 1998.

[b] Numbering is according to the cDNA starting at the A in the start codon (Antonarakis, 1998) (Genbank Accession No. Z13009).

Comparison between E-cadherin Mutational Status and the Histologic Type of the Cases

In the group of 10 "pure" intestinal carcinomas, no mutations were found. Of the 10 "pure" diffuse carcinomas, we found mutations in seven cases (70.0%) (Table 2).

E-Cadherin mutations were detected in five of the six cases of mixed gastric carcinoma (83.3%) (Table 2). In four cases, we detected mutation of the E-cadherin gene restricted to the diffuse component of the tumors (Fig. 1) (cases 11, 13, 14, and 15). In case 16, we found two different mutations in the distinct histologic components of the tumor: a missense mutation in the intestinal type component; and a splice site mutation in the diffuse type component (Table 2).

A significant relationship was found between the diffuse histotype (both in "pure" and in mixed carcinomas) and E-cadherin mutations ($p < 0.0001$). In 16 samples of the diffuse type (10 "pure" diffuse carcinomas and 6 diffuse components of mixed carcinomas), we found 12 samples (75.0%) presenting E-cadherin mutations. In contrast to this, only 1 of the 16 samples of the intestinal type displayed an E-cadherin mutation, corresponding to the intestinal component of a mixed carcinoma.

LOH Analysis

All cases included in the present study were informative for at least one of the two microsatellite markers. One case of the 26 (3.8%) showed LOH for marker D16S301 (data not shown). In this case, a missense mutation in one of the calcium binding sequences of the gene was also found (Table 2, case 3). Regarding the polymorphisms detected, the same pattern was observed in tumoral and matched constitutional DNA.

Discussion

We detected E-cadherin gene mutations in 46.2% of gastric carcinomas. By sequencing analysis, we identified 15 mutations, mostly of the missense type. In a previous study that used a strategy based on reverse transcriptase (RT)-PCR, Becker et al (1994) reported E-cadherin gene mutations in 50% of gastric carcinomas of the diffuse type and described a high frequency of exon skipping of E-cadherin gene affecting exons 8 and 9.

In accordance with the results of Becker et al (1994), we found that mutations were clustered in the central region of the E-cadherin gene (exons 7 to 12). This region codes for the five extracellular cadherin domains of the protein, each containing two highly conserved calcium binding motifs (Kemler, 1993), that play a key role in the adhesion process (Overduin et al, 1995; Shapiro et al, 1995). Seven of the 10 missense mutations detected in the present study occurred in

Table 2. Summary of the Results Regarding E-cadherin Mutations in 16 Gastric Carcinomas with "Pure" Diffuse and Mixed Histotypes

Case	Histologic type	Mutation			
		Exon No.	Codon	Nucleotide change[a]	Predicted protein change
1	Diffuse	—	—	—	—
2	Diffuse	8	353	1057G→T	E353X (stop)
3	Diffuse	8	370	1108G→C	D370H (missense)
4	Diffuse	10	479	1436A→G	D479G (missense)
5	Diffuse	8	343	1027delC	Frameshift
		9	402	1204G→A	D402N (missense)
6	Diffuse	12	581	1742T→C	L581P (missense)
7	Diffuse	Intron 7	—	1009-1G→A	In frame deletion
		8	347	1040C→T	A347V (missense)
8	Diffuse	—	—	—	—
9	Diffuse	—	—	—	—
10	Diffuse	7	334	1000G→C	D334H (missense)
11	Mixed				
	D	8	369	1105A→G	N369D (missense)
	I	—	—	—	—
12	Mixed				
	D	—	—	—	—
	I	—	—	—	—
13	Mixed				
	D	Intron 7	—	1009-1G→A	In frame deletion
	I	—	—	—	—
14	Mixed				
	D	9	400	1198G→T	D400Y (missense)
	I	—	—	—	—
15	Mixed				
	D	8	370	1108G→C	D370H (missense)
	I	—	—	—	—
16	Mixed				
	D	Intron 7	—	1009-1G→A	In frame deletion
	I	8	365	1093G→C	V365L (missense)

[a] Numbering is according to the cDNA starting at the A in the start codon (Antonarakis, 1998) (Genbank Accession No. Z13009).
D, diffuse component of mixed carcinoma; I, intestinal component of mixed carcinoma.

one of the sequences coding for calcium motifs, being expected to impair cell-cell adhesion by interference with the calcium dependent cell adhesion zipper (Shapiro et al, 1995). In fact, it was previously shown that conservative point mutations within the N-terminal calcium binding pocket are enough to abolish cell-cell adhesion (Ozawa et al, 1990). The remaining three missense mutations led to two conservative substitutions and one nonconservative substitution. The latter, leading to the replacement of a leucine by a proline residue (case 6), probably causes major alterations in the protein structure. The two conservative substitutions, corresponding to nonconserved sequences of the gene, are not expected to produce severe alterations in the resulting protein structure and, consequently, in its function. One of these mutations occurred in case 7 together with a potentially inactivating splice site mutation, and the other was restricted to the intestinal component of a mixed carcinoma (case 16). On the other hand, it is interesting that some missense mutations seem to have their major effect by induction of aberrant splicing (Oda et al, 1994).

The nonsense mutation and the frameshift deletion, resulting in premature downstream stop codons, are expected to produce truncated peptides lacking the transmembrane and the cytoplasmic β-catenin binding domains. In three cases, we found a mutation affecting intron 7 acceptor splice site. Unavailability of corresponding mRNA prevented us from further analyzing the consequences of such alteration at the transcription level. Mutations occurring in the same splice site were previously described by Berx et al (1996) and by Becker et al (1994), resulting in exon 8 skipping.

In the classic two-hit model of tumor suppressor gene inactivation, mutations in one allele are accompanied by deletion of the remaining normal allele. Our results appear to challenge this model because LOH of 16q22.1 was found in only 1 of the 12 cases presenting mutation of the E-cadherin gene. Noteworthy, in case 5, two different inactivating mutations were identified, thus fulfilling also the two-hit inactivation model. These findings contrast with those of Berx et al (1996) in lobular carcinoma of the breast, showing

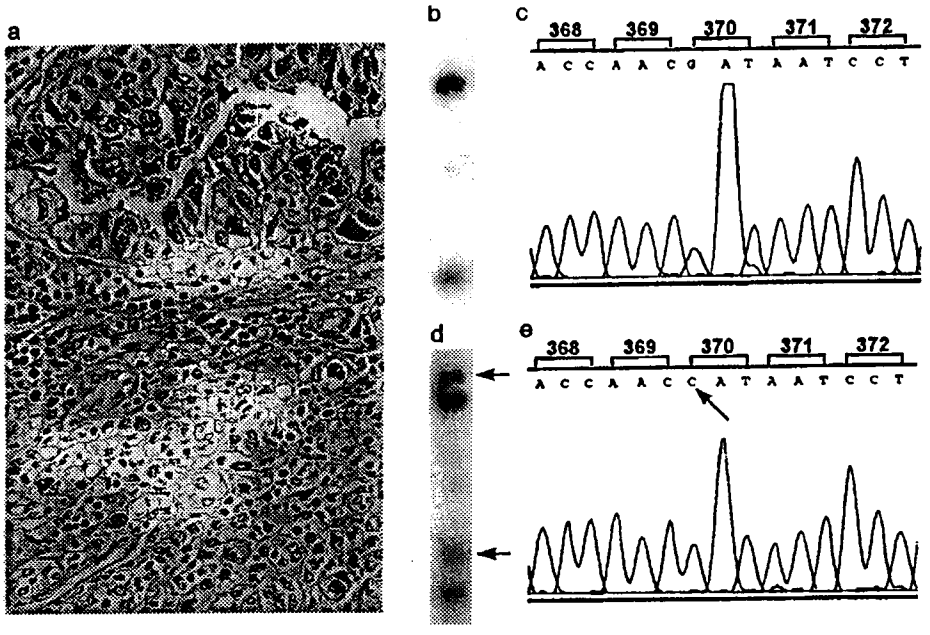

Figure 1.
E-Cadherin gene mutation restricted to the diffuse component in a mixed gastric carcinoma (case 15). a, Hematoxylin and eosin staining of mixed gastric carcinoma presenting adjacent intestinal (top) and diffuse (bottom) histologic components (original magnification, ×140). b, Exon 8 PCR/single-strain conformation polymorphism (SSCP) analysis of intestinal component showing a regular band pattern. c, Sequencing analysis of the bands represented in b showing a normal E-cadherin sequence corresponding to codons 368 to 372. d, In the diffuse component, two band shifts (*arrows*) were found by PCR/SSCP analysis of exon 8, in addition to the regular bands. e, Sequencing analysis of the abnormal bands represented in d, revealed a missense mutation (1108G→C; D370H) in the E-cadherin gene (*arrow*).

that 93% of cases with E-cadherin gene mutation displayed also LOH. Alternative inactivation mechanisms of the remaining normal allele may include specific allelic exclusion (Becker and Hofler, 1995) or alterations at the gene promoter level (Hennig et al, 1995). Methylation within the promoter or the 5′-CpG island of the E-cadherin gene has been shown in breast and prostate carcinomas (Graff et al, 1995; Hennig et al, 1995; Yoshiura et al, 1995). Additionally, one can speculate that the mutation of one allele may result in a dominant-negative effect over the remaining normal allele. Cadherins exist at the cell surface as dimers linked by interactions at each cadherin domain (Shapiro et al, 1995). One may admit that a mutated E-cadherin protein may still bind to wild-type protein leading to a nonfunctional E-cadherin complex.

In the present series, E-cadherin mutations occurred in 70% of "pure" diffuse gastric carcinomas and were not detected in "pure" intestinal carcinomas. This very strong association between E-cadherin mutations and the diffuse histotype is in agreement with the results of Becker et al (1994), who reported mutations of the E-cadherin gene in 50% of diffuse gastric carcinomas and its absence in intestinal type carcinomas. Furthermore, E-cadherin mutations were detected in gastric carcinoma cell lines with a diffuse phenotype (Oda et al, 1994) and in families of patients with diffuse gastric carcinoma (Gayther et al, 1998; Guilford et al, 1998). In mixed gastric carcinomas, we observed that inactivating E-cadherin mutations were restricted to the diffuse component of the tumors in five of six cases. This finding suggests the existence of genotypically divergent tumor clones in mixed gastric carcinoma.

Altogether, these results suggest that inactivation of the E-cadherin gene determines the diffuse histotype of gastric carcinoma. This putative cause-effect relationship between E-cadherin inactivation and the diffuse histotype is challenged by the existence of a small percentage of cases presenting diffuse histotype without detectable E-cadherin mutations (cases 8 and 9 and diffuse component of case 12). In such cases, one has to admit that the adhesion of neoplastic cells is disrupted by an alteration at another level of the cell adhesion system. In fact, E-cadherin is only one of the constituents of a complex cell adhesion system, and its function is dependent on the interaction of its conserved cytoplasmic domain (C-terminal) with the cytoskeleton by means of molecular complexes involving α-, β-, and γ-catenin (Kemler, 1993). Alterations in the associated cytoplasmic molecules, such as the catenins, are thought to impair cell adhesion in tumors exhibiting normal E-cadherin expression (Bukholm et al, 1998; Jawhari et al, 1997; Shimoyama et al, 1992; Smith and Pignatelli, 1997). Furthermore, recent data indicate that phosphorylation of tyrosine residues of β-catenin, by means of epidermal growth factor receptor and/or c-erbB2 activation, may inhibit the assembly of the E-cadherin-catenin-cytoskeleton complex leading to the disruption of the adhesion system (Vermeulen et al, 1996). Alternatively, we cannot rule out the possibility that some mutations were not detected by the present

analysis, although SSCP is expected to be highly sensitive.

Summing up, we observed that E-cadherin inactivation is significantly related with the diffuse histotype in gastric carcinomas, not only in "pure" diffuse carcinomas but also in the diffuse component of mixed tumors. To the best of our knowledge, this is the first report advancing a genetic basis for the phenotypic divergence of mixed gastric carcinomas.

Materials and Methods

Tissue Material

Surgical specimens from 26 gastric carcinomas resected and diagnosed at Hospital S. João/Medical Faculty and IPATIMUP, Porto, Portugal, were studied. According to Laurén's classification (Laurén, 1965), cases were classified as intestinal (n = 10) and diffuse (n = 16). Six cases, classified according to the predominant histologic type as diffuse carcinomas, also displayed foci of intestinal carcinoma. These cases were subclassified, according to the classification proposed by Carneiro et al (1995), as mixed carcinomas.

Tumor Microdissection and Genomic DNA Extraction

Genomic DNA extraction was carried out in serial sections of formalin-fixed, paraffin-embedded tumor material. To reduce contamination from nontumor surrounding tissue, we performed microdissection of the tumor areas in four 10-μm serial sections, after examination of hematoxylin and eosin–stained serial sections. Microdissection was also used to obtain separate samples of distinct histologic components of mixed carcinomas. Microdissected tissues were depleted of embedding material by incubation with xylene (5 minutes), ethanol (5 minutes), and acetone (1 minute). After each incubation step, the material was recovered by centrifugation at 13,000 ×g (1 minute). Genomic DNA was retrieved from the resulting material by using standard phenol/chloroform extraction.

Mutation Screening by PCR/SSCP

SSCP analysis was performed by [^{32}P]dCTP radioactive PCR amplification of the complete E-cadherin coding sequence and primers were designed to amplify individually each E-cadherin exon, including exon/intron boundaries. Primer sequences and PCR conditions were based on those reported by Berx et al (1995), except for exons 2, 4, and 5, which are shown in Table 3, along with optimal annealing temperatures and expected product size information. Cycling conditions were 30 seconds at 94° C, 30 seconds at the appropriate annealing temperature, and 1 minute at 72° C for 35 cycles. PCR negative controls were performed by replacing the template DNA with water. Before electrophoresis, PCR reaction products were diluted 1:1 with loading buffer (95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol), denatured at 99° C for 2 minutes and cooled on ice for 5 minutes. Electrophoresis of the denatured PCR products was carried out in nondenaturing gels (5% polyacrylamide with 2% cross-linking), and run at 3 W, 8° C. Gels were blotted onto 3MM Whatman paper, dried, and exposed to x-ray film at room temperature. All cases presenting band shifts were submitted to a second analysis (new PCR amplification and SSCP analysis), and only reproducible bands were considered.

Sequencing Analysis

Abnormal bands, as well as corresponding normal bands, detected by SSCP, were recovered from the gels and submitted to nonradioactive PCR reamplification with the original set of primers. Reamplification products were purified and sequenced by using the ABI Prism Dye Terminator Cycle Sequencing kit (Perkin-Elmer, Foster City, California) and an ABI Prism 377 DNA Sequencer (Perkin-Elmer). Sequencing was performed on both strands, by using the original primers.

LOH Analysis

The LOH study was done on the same tumor DNA used for SSCP analysis and on matched constitutional DNA. Radioactive PCR amplification of microsatellite markers D16S265 and D16S301 (Table 1) (Human Gene Mapping Kit, Isogen, Amsterdam, Netherlands) was performed according to the procedures de-

Table 3. Primer Sequences and PCR Conditions for PCR/SSCP and LOH Analysis

Analysis	Primer name	Primer sequence (5′ - 3′)	Product size (bp)	T_A (°C)
PCR/SSCP	2F	TCACCCGGTTCCATCTAC	198	58
	2R	TTCCAACCCCTCCCTACT		
	4F	CTTGTTCCTCATCTTCTTTC	237	58
	4R	CCCTTTCTCTCCTTGGTACT		
	5F	GTTGGGATCCTTCTTTACTA	256	58
	5R	AAATCCTGGGTGGATGTTAC		
LOH	D16S265F	CCAGACATGGCAGTCTCTA	89–117	58
	D16S265R	AGTCCTCTGTGCACTTTGT		
	D16S301F	GATCCTAAGGACAAATGTAGATGCTCT	142–152	58
	D16S301R	AGCCACTTCCCAGAACTTGGCTTCC		

F, forward primer; R, reverse primer; T_A, annealing temperature; SSCP, single-strain conformation polymorphism; LOH, loss of heterozygosity.

scribed above for PCR/SSCP. PCR products were run in a denaturing gel (6% polyacrylamide with 5% crosslinking) and exposed to x-ray film at room temperature. Polymorphisms of the E-cadherin gene were used as intragenic markers for the detection of LOH at the E-cadherin locus.

Statistical Analysis

The statistical analysis of the results was performed by using the Pearson χ^2 test. Differences were considered to be significant at $p < 0.05$.

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