Double immunostaining for p53 and molecular chaperone hsp72/73 in gastric carcinoma

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Abstract

Aims—To examine the relation between the expression of p53 protein and the chaperone heat shock protein (hsp)72/73 in a population at high risk for gastric carcinoma, using single and double immunohistochemistry, and to compare the expression of these two proteins with clinicopathological features.

Methods—Monoclonal antibodies were used to investigate the expression of p53 protein and hsp72/73 in 46 human gastric carcinomas. A double immunohistochemical technique was used in cases that showed p53/hsp72/73 coexpression.

Results—p53 immunoreactivity was present in 11 tumours (24%), and hsp72/73 immunostaining was observed in 22 cases (48%). p53 expression was observed as nuclear staining in tumoral cells. hsp72/73 expression was demonstrated mainly as cytoplasmic staining, but six tumours also showed focal weak nuclear staining. Seven cases showed p53 and hsp72/73 coexpression with immunoreactivity for both proteins in the same neoplastic cells, three of them with focal areas of nuclear coexpression. p53 expression was seen more frequently in cases that showed a high intensity (+++) of hsp72/73 staining. No significant association was observed between the expression of the two proteins and clinicopathological features.

Conclusions—More than half of our cases may have some impairment in p53 protein growth suppressive function, as a result of p53 gene alterations or complex formation. The positive correlation between p53 expression and intensity of hsp72/73 supports the postulate of a p53 regulating function for the chaperone hsp72/73. A high intensity of hsp72/73 immunohistochemical staining could be used as an indirect marker of p53 gene abnormalities.

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that interfere with normal control of cell growth and differentiation. Tumour suppressor genes are normal cellular genes, which when inactivated lead to a dysregulation of both the cell cycle and apoptosis. These disturbances may facilitate the development of neoplasia.⁴ Cancer cells differ from normal cells in many important characteristics including loss of differentiation, increased invasiveness, and decreased drug sensitivity. There is evidence that conversion of normal epithelial cells to cancerous cells is a multistep process that requires the accumulation of multiple gene abnormalities affecting DNA repair genes, oncogenes, and tumour suppressor genes.^{5,6} Gastric cancer is no exception and it displays multiple gene alterations involving oncogenes, growth factor or cytokine genes, cell cycle regulatory genes, tumour suppressor genes, and cell adhesion molecule genes. These gene alterations may lead to genetic instability.⁷

The p53 tumour suppressor gene is located on the short arm of human chromosome 17 at position 17p13.1. This gene is composed of 11 exons, the first of which is non-coding, and is localised 8-10 kb away from exons 2-11.^{8,9} The product of p53 gene is a 375 amino acid (with a molecular mass of ~ 53 kDa) nuclear phosphoprotein, which was first identified as a cellular protein in 1979 because it formed a tight complex with the SV40 large T antigen.¹⁰ The p53 protein was found in very low quantities in normal cells, but large quantities of p53 (5-100-fold) could be detected in transformed cells in culture and in human tumours. Loss of normal p53 function could be achieved in a variety of ways including genetic changes in the p53 gene (such as mutation, deletion, or structural rearrangements), formation of protein complexes with viral oncoproteins (such as SV40 large T antigen, adenovirus E1B, and papillomavirus E6), and binding to cellular proteins (such as MDM2).¹¹ p53 mutations are now recognised as being one of the most common cancer related genetic changes seen at the gene level.⁶ Mutant p53 protein is thought to act by formation of pseudohomodimers of wild-type and mutated p53 which abrogate the growth suppressive activity of the normal protein.¹² A further loss of proliferative control may occur when the wild-type allele is deleted from the cells.¹³ However, genetic alterations of the p53 gene are not the only mechanism by which the p53 protein can be stabilised. The normal protein can be stabilised by the action of viral and cellular gene products.¹¹ There is evidence that mutated and probably wild-type

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Gastric cancer is one of the most prevalent malignancies in the world, but there is substantial variation in the incidence and mortality rate of the disease among different regions or countries.^{1,2} Chile is one of the countries with the highest reported frequencies.^{1,3} There is increasing evidence that cancers arise as the result of an accumulation of genetic alterations

Figure 1 Nuclear p53 immunoreactivity in a moderately differentiated gastric adenocarcinoma. Streptavidin–biotin peroxidase reaction with haematoxylin counterstaining. (Original magnification $\times 400$.)

Figure 2 Cytoplasmic hsp72/73 immunoreactivity in a neoplastic gastric epithelium. Streptavidin–biotin peroxidase reaction with haematoxylin counterstaining. (Original magnification $\times 1000$.)

p53 protein may form complexes with some heat shock proteins (hsp), such as hsp70 isoforms.¹⁴

Heat shock proteins are produced after cell exposure to stressful conditions such as temperature shock, chemicals, and other forms of environmental stress.^{15–16} They act as molecular chaperones that bind to denatured or inappropriately folded proteins, assisting their correct non-covalent assembly, although they are not components of the functional assembled structures.¹⁷ hsp70 concentrates in nuclei during heat shock and returns to the cytoplasm when the shock is removed. Although hsp70 family members bind to mutant p53 in transformed cells, it has been proposed also that the formation of complexes with wild-type p53 protein may regulate or interfere with its function.¹⁴

This work evaluates the expression of p53 protein and hsp72/73 in a high risk population for gastric carcinoma, using single and double

immunohistochemistry, and compares it with clinicopathological features.

Methods

A random sample of 46 surgically resected gastric carcinoma patients was selected from our registry of tumoural diseases. Representative inclusions of formalin fixed, paraffin wax embedded tissue were obtained from the archives of the department of pathology, Temuco's Regional Hospital, Temuco, Chile. The sample included five early gastric cancers and 41 advanced gastric carcinomas. The mean age of the patients was 62 years (range 34–89) and the sex ratio was 1.4:1 (male:female). Surgical specimens were processed according to the general rules of the Japanese Gastroenterology Society,¹⁸ and the tumours were classified according to WHO histological criteria¹⁹ and Lauren's histological types.²⁰

IMMUNOHISTOCHEMISTRY

p53 and hsp72/73 expression was detected using commercially available monoclonal antibodies. Immunohistochemical studies were performed by means of streptavidin–biotin methodology. Briefly, 4 μ m thick paraffin wax sections were dewaxed and subjected to microwave antigen retrieval by immersion in citrate buffer (pH 6.0) at 800 W. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 minutes and sections were then treated with 20% normal goat serum for 20 minutes. Antigen immunoreactivity was detected by incubation with primary antibodies directed against p53 at 1/100 dilution (clone DO1; Oncogene Science, New York, USA), and hsp72/73 at 1/100 dilution (clone W27; Oncogene Science), for one hour at room temperature, according to the manufacturer's instructions. Sections were then washed and incubated with a biotinylated secondary antibody (Multilink; Biogenex, San Ramon, California, USA). After incubation with streptavidin–biotin peroxidase complex (Biogenex) for 20 minutes, sections were incubated in 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemicals, St Louis,

Figure 3 Weak nuclear hsp72/73 immunostaining in a moderately differentiated gastric adenocarcinoma. Streptavidin–biotin peroxidase reaction with haematoxylin counterstaining. No significant association was observed between the expression of the two proteins and clinicopathological parameters such as tumour location, histological type, histological grade, Lauren's type, and nodal status (table 1). However, cardiac tumours were more frequently positive for hsp72/73 than distal tumours ($p = 0.08$), mainly in the p53 negative subgroup. In addition, hsp72/73 was observed more frequently in male patients ($p = 0.06$) and in early gastric carcinomas ($p < 0.15$). (Original magnification $\times 1000$.)

Figure 4 p53/hsp72/73 double immunostaining in a moderately differentiated gastric adenocarcinoma. Note the nuclear p53 immunoreactivity (brown) and the cytoplasmic and nuclear hsp72/73 immunostaining (red). Streptavidin–biotin peroxidase reaction (brown) and streptavidin–biotin alkaline phosphatase reaction (red) with haematoxylin counterstaining. (Original magnification × 1000.)

Figure 5 p53/hsp72/73 double immunostaining in lymphatic tumour permeation. Note nuclear p53 (brown)/hsp72/73 (red) coexpression (arrows). Streptavidin–biotin peroxidase reaction (brown) and streptavidin–biotin alkaline phosphatase reaction (red) with haematoxylin counterstaining. (Original magnification × 1000.)

Missouri, USA). Finally, the sections were counterstained weakly with haematoxylin. Positive controls were sections from breast carcinoma known to express p53 oncoprotein and hsp72/73. Negative controls were included by incubation and development of sections using only normal blocking serum without incubation in primary antibody.

DOUBLE IMMUNOHISTOCHEMISTRY
All cases that showed both p53 and hsp72/73 immunostaining were subjected to double immunostaining by means of a sequential double labelling technique, using two different detection systems. Briefly, sections were stained first for p53 protein, using the streptavidin–biotin peroxidase detection system, and visualisation with DAB (brown) as described previ-

ously. Sections were then washed and stained for hsp72/73 using the streptavidin–biotin alkaline phosphatase detection system and visualisation with fast red (red).
All immunostained slides were analysed and scored in a blind fashion by two different observers. Staining intensity was graded according to the numbers of positive cells as follows: fewer than 5% of tumour cells positive (–); 5–25% of tumour cells positive (+); 26–50% of tumour cells positive (++); greater than 50% of tumour cells positive (+++). Statistical analysis was undertaken using the Student’s *t* test, Fisher’s exact test, and Pearson’s correlation test.

Results
p53 immunoreactivity was present in 11 (24%) tumours and hsp72/73 immunostaining was observed in 22 (48%) cases (table 1). p53 expression was seen as nuclear staining in tumoural cells (fig 1). Nuclear positivity was not seen in the non-cancerous mucosa adjacent to the carcinoma. hsp72/73 expression was demonstrated mainly as a fine granular cytoplasmic staining pattern in neoplastic cells (fig 2). Six tumours (13%) also showed focal weak nuclear staining (fig 3). All cases that showed nuclear hsp72/73 expression were also p53 positive. Cytoplasmic hsp72/73 was also detected in non-neoplastic mucosa adjacent to the tumours, mainly in intestinal metaplasia areas.
Seven cases (15%) showed p53 and hsp72/73 coexpression with immunoreactivity for both proteins in the same neoplastic cell,

Table 1 Clinicopathological features, p53 and hsp72/73 immunoreactivity in human gastric carcinoma

Clinicopathological features	p53	hsp72/73
Sex		
Male	7/27 (26)	16/27 (59)*
Female	4/19 (21)	6/19 (32)
Age		
< 50 years	0/7	2/7 (29)
≥ 50 years	11/39 (28)	20/39 (51)
Tumour location		
Cardial third	4/17 (24)	11/17 (65)
Medial third	3/9 (33)	4/9 (44)
Antral third	4/20 (20)	7/20 (35)
Wall stage		
Early carcinoma	2/5 (40)	4/5 (80)
Advanced carcinoma	9/41 (22)	18/41 (44)
Histological type		
Tubular adenocarcinoma	9/33 (27)	15/33 (45)
Signet ring cell carcinoma	1/9 (11)	5/9 (56)
Mucinous adenocarcinoma	0/2	1/2 (50)
Undifferentiated carcinoma	1/2 (50)	1/2 (50)
Histological grade		
Well/moderately	2/13 (15)	8/13 (62)
Poor	9/33 (27)	14/33 (42)
Lauren type		
Intestinal	5/21 (24)	11/21 (52)
Diffuse	6/25 (24)	11/25 (44)
Lymph node status		
Negative	2/10 (20)	4/10 (40)
Positive	8/34 (24)	17/34 (50)

Values are n (%). *p = 0.06.

Table 2 hsp72/73 immunostaining intensity according to p53 expression in human gastric carcinoma

p53	hsp72/73			
	(–)	(+)	(++)	(+++)
Negative	20 (83)	5 (83)	9 (82)	1 (20)
Positive	4 (17)	1 (17)	2 (18)	4 (80)

Values are n (%).
p < 0.02, Pearson’s correlation value: r = +0.31.

three of them (7%) with focal areas of nuclear coexpression (figs 4 and 5). An association between intensity of hsp72/73 and p53 expression was seen (table 2). Eighty per cent of cases showing high (+++) hsp72/73 intensity were p53 positive and only 17% of tumours that showed a lower intensity or were negative for hsp72/73 were p53 positive ($p < 0.02$; Pearson's correlation value: $r = +0.31$). A total of 26 cases (57%) showed expression of p53 and/or hsp72/73.

Discussion

Several gene alterations have been found to be important in the upper digestive tract including those affecting multiple oncogenes, tumour suppressor genes, cell cycle regulator genes, and DNA repair genes.^{21, 22} Altered expression and amplification of the c-met gene, inactivation of the p53 gene, and abnormal CD44 transcripts are common events in gastric carcinoma.²¹ Tahara has demonstrated that gene changes differ in relation to the two histological types, because intestinal or differentiated types and diffuse or undifferentiated types of carcinoma may develop by different genetic pathways.^{7, 21} Genetic alterations of the p53 gene are found frequently in both types of gastric carcinomas. These alterations lead to p53 protein overexpression and allow the formation of complexes with some heat shock proteins, such as hsp70 isoforms.^{11, 14}

In the present study, although we found p53 expression in only 24% of cases, almost half of the tumours showed hsp72/73 expression. Thus, 57% of cases may have some impairment of p53 protein growth suppressive function, as a result of p53 gene alteration or complex formation. In cases that were negative for p53 protein and positive for hsp72/73, the chaperone may have exerted a wild-type p53 blocking effect that could also have given a proliferative advantage to malignant cells. Both the observation that hsp72/73 is expressed in non-neoplastic mucosa (particularly in metaplastic areas) and the trend towards higher chaperone expression in early gastric carcinomas suggest that hsp72/73 induction may be related to early events in the multistep carcinogenic model proposed by Correa.²³ This may represent an early non-genetic p53 protein dysfunction. Polymorphonuclear leucocyte chemotaxis and activation by *Helicobacter pylori* (with their oxidative effects) may play a role in the early stages of gastric carcinogenesis. These are known causes of DNA damage and hypothetically could induce mutations in replicating gastric epithelial cells.²⁴ To our knowledge, there are no studies on *H. pylori* status and hsp70 isoform expression in gastric epithelial lesions. These hypotheses must be studied by means of genetic analysis of p53 genes in relation to hsp expression analysis, by cell proliferation assessment, and by *H. pylori* status assessment in early gastric carcinoma and preneoplastic lesions.

The main finding of our study is that of the positive correlation of p53 expression and intensity of hsp72/73 staining, concordant with the observation that hsp72/73 binds to mutant

p53 protein. Thus, hsp72/73 expression may be used as an indirect marker of p53 gene abnormalities. This association also suggests that the unfavourable prognostic outcome described for hsp expression in some tumours²⁵ may be related to the alteration of associated p53. Thus, hsp chaperones could be a p53 dependent prognostic factor. In the literature, Elledge *et al* found no correlation between hsp70 expression and p53 accumulation in breast carcinoma,²⁶ whereas Chant *et al* did find such an association in acute myeloid leukemia.²⁷ Recently, some additional carcinogenic hsp mediated mechanisms have been postulated. hsp chaperones may protect certain tumour cells from tumour necrosis factor mediated cytotoxicity by interfering with the signal transduction pathway leading to the activation of phospholipase A₂.²⁸

In conclusion, the accumulation of hsp72/73 may be related to the early stages of gastric carcinogenesis. The association of p53 and hsp72/73 accumulation supports the postulate of a p53 regulating function for the chaperone hsp72/73.

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