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Establishment and characterization of two cell lines derived from human diffuse gastric carcinomas xenografted in nude mice

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Abstract Two human diffuse gastric carcinoma cell lines were established in vitro from xenografted tumours serially passaged in nude mice. Of 12 primary diffuse gastric carcinomas, 7 were successfully xenografted in nude mice (58.3%). Short-term primary cultures were achieved in all the xenografted lines. However, only 2 of the 7 short-term primary cultures were established as long-term cultures (GP202 and GP220). GP202 cells are larger than GP220 cells, show less abundant intercellular junctions at the ultrastructural level and grow in culture as a compact thin monolayer. The GP220 cells grow preferentially in small clusters attached to the monolayer, with a subpopulation of floating cells. Both lines have cells containing small mucin vacuoles in the cytoplasm and cells displaying a typical signet-ring shape. GP202 cells grow as solid tumours in nude mice but GP220 cells do not give rise to tumours. The flow cytometry and karyotype analysis showed aneuploidy in GP202 cells, with many numerical and structural chromosomal abnormalities, and diploidy in GP220 cells, with several structural chromosomal abnormalities. The CDw75 and Tn antigens are more prominently expressed in GP202 cells than in GP220 cells. T antigen is only expressed in GP202 cells, whereas only GP220 cells express EGFR. Sialosyl-Tn is not expressed in either of the cell lines. The gastric cancer cell lines described in this paper represent a valuable addition to the small number of diffuse gastric cancer cell lines currently available and also provide a good model for further in vitro and in vivo studies of gastric carcinogenesis.

Key words Gastric carcinoma · Diffuse type gastric carcinoma · Cancer cell lines · Gastric carcinoma cell line · Nude mice

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Introduction

Despite the decrease in gastric cancer incidence rates in recent decades, gastric cancer is still the leading cause of cancer death in Portugal [13] and the second most common cause of cancer death worldwide, after lung cancer [23].

The search for the molecular mechanisms involved in gastric carcinogenesis would be facilitated if a panel of different tumour cell lines representing the different tumour phenotypes of gastric carcinoma could be developed. A number of well-characterized cell lines established from intestinal type gastric carcinomas are currently available. In contrast to this, only a few diffuse-type gastric carcinoma cell lines have been successfully established, mostly from metastatic deposits and ascitic fluid [12, 17, 33, 35, 37]. The reason why gastric carcinomas of the diffuse type are extremely difficult to grow in culture [26, 31] and in nude mice [34] is not understood. Moreover, although most diffuse gastric carcinomas are diploid or near-diploid [9], all of the four diffuse gastric carcinoma cell lines on record are aneuploid [12, 33, 37, 38, 40], suggesting that there is a bias in the selection of in vitro-growing cells.

Because most cultures of diffuse type gastric carcinoma simply become overgrown by stromal fibroblasts, it is postulated that the development of this type of tumour may be dependent on growth-promoting factors produced by stromal cells [39]. This dependence might contribute to the difficulties of growing tumour cells of this type free of fibroblasts in tissue culture [10].

In this study, we report the successful establishment and characterization of two gastric carcinoma cell lines derived from human primary tumours of the diffuse type, heterotransplanted and serially passaged in nude mice. Of the seven diffuse gastric carcinomas successfully xenografted in nude mice, 100% were established as primary cultures, but only two (28.5%) as long-term cultures. The cell lines have been studied with respect to growth properties, morphology, karyotype, and expression of a panel of antigens that included cytokeratins,

gastrointestinal epithelial antigens, proteolytic enzymes and mucin-type antigens. Their tumorigenicity has been tested both in soft agar and in nude mice.

Materials and methods

Twelve primary diffuse-type gastric carcinomas were selected for the establishment of xenografts in the nude mice. Frozen sections were prepared in each case and areas exclusively composed of isolated cells were selected for the xenografts.

For heterotransplantation, mice of the N:NIH(s)II-*nu/nu* strain [2] were reared from stocks imported from the Animal Unit, Medical School, University of Cape Town, S.A. For the experiments we used 4- to 5-week-old progeny of *nu/*+mothers and *nu/nu* fathers.

Xenografted tumours with a volume of approximately 1500 mm^3 were resected under aseptic conditions and divided into representative portions for histology, immunohistochemical analysis, flow cytometry, cytogenetic analysis and passage in vivo. One fragment with a volume of approximately 6 mm^3 was reimplanted subcutaneously into each of three nude mice to preserve the xenografted tumour lines.

In vitro cell lines were established from two human tumours (GP202 and GP220). The GP202 cell line was derived from a primary gastric carcinoma in a 53-year-old man. The tumour was localized in the body region invaded the subserosa (T2), had nodal metasases in perigastric lymph nodes (N2) and displayed a predominantly solid pattern of growth with signet-ring cells. The GP220 cell line was derived from a primary gastric carcinoma in an 85-year-old man. The tumour was localized in the antrum, penetrated the serosa (T3), had nodal metastases (N2) and displayed a diffuse pattern of growth with numerous signet-ring cells.

The cell lines were established after being passaged as xenografts. Xenografted tumours in passages 4 (GP202) and 2 (GP220), with a volume of approximately 1500 mm^3, were removed aseptically. Single cell suspensions were prepared by mechanical disaggregation. The resulting suspensions were filtered through a 30-mm wire mesh to remove cell aggregates and then seeded in tissue culture flasks (TPP). The culture medium, RPMI 1640 medium (Gibco) with 25 mM HEPES and Glutamax-1, was supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin. The flasks were maintained in a 37°C incubator with a humidified atmosphere of 5% CO_2 in air. When cultures became confluent, the cells were treated with 0.05% trypsin/0.02% EDTA in a PBS solution and subcultured. Murine fibroblasts contaminating the initial cultures were removed by differential trypsinization [3] to obtain a pure tumour cell population. Culture passages were performed weekly. Cell cultures in passages 4–8 were found to be free from murine fibroblasts, via both the immunohistochemical detection of cytokeratins in the cultured cells and verification of the absence of murine metaphases in the cytogenetic analysis of dividing cells.

For the study of morphology, cells were examined using an Olympus inverted microscope after staining with crystal violet (1% crystal violet in 100% methanol).

For soft agar and growth kinetics experiments, tumour cells were harvested from subconfluent monolayer cultures by treatment with 0.05% trypsin/0.02% EDTA in PBS, washed, and counted in a Neubaur chamber. Cells (n=1–3×10^3) were plated on 30-mm-diameter dishes containing 0.2% agarose and grown at 37°C and with 5% CO_2 in air. For growth kinetics, tumour cells were seeded on 24-well culture plates at a fixed cell number on day 0, and the number of cells per well was measured as a function of time. A growth curve was calculated by logarithmic regression analysis and the doubling time was estimated from this curve.

For in vivo experiments on solid tumour growth, 10^6–10^8 tumour cells were injected subcutaneously into the superscapular region of five nude mice. For these experiments only cell suspensions with viability greater than 90% were used. Mice were examined and their tumours were measured once a week in three dimensions, and tumour volume was calculated using the formula: V=length×(width)^2×1/2. Tumour-doubling time (Td) was calculated from the logarithmic portion of the growth curve.

Fragments from the patients' primary tumours, xenografts (several passages) and cell lines (several passages) were studied by transmission electron micoscopy, flow cytometry and immunohistochemistry. For electron microscopy a Jeol 100CX II electron microscope was used. Flow cytometry was performed using an EPICS/CS flow cytometer (Coulter Electronics, Hialech, Fla.) fitted with a single INNOVA 90 argon ion laser [9]. Immunohistochemistry was performed as previously reported [6]. Primary antibodies, their sources, dilutions and specificities are detailed in Table 1.

In the two cases that originated cell lines (GP202 and GP220) cytogenetic analysis was performed both in xenografts and in the cell lines, as previously described [1, 16].

Table 1 Primary antibodies

Antigen/clone	Nature of the molecule	Source[a]	Dilution
CK 7/RCK105	Cytokeratin 7	A	Undiluted[b]
CK 8/M20	Cytokeratin 8	A	1:5
CK 10/RKS E60	Cytokeratin 10	A	1:5
CK 13/1C7	Cytokeratin 13	A	Undiluted[b]
CK 18/RCK106	Cytokeratin 18	A	1:5
CK 19/RCK108	Cytokeratin 19	A	1:50
Cathepsin D	Lysosomal enzyme involved in intracellular protein turnover	B	1:50[b]
Collagenase IV/CA7 19E3C	Human metalloproteinase	C	1:75
MUC1/HMFG1	MUC1 mucin	D	1:100
CEA/12–240–2	Carcinoembryonic antigen	E	1:320
CDw75/HH2	Sialylated carbohydrate generated by the β-galactosyl α2,6 sialyltransferase	F	1:100
Tn/HB-Tn1	GalNAcα1-O-serine/threonine	G	1:5
T antigen/HB-T1	Galβ1–3Gal-N-Acα1-O-serine/threonine	G	1:10
EGFR	Tyrosine kinase receptor for EGF and TGF	H	1:75
Sialosyl-Tn/HB-STn1	NeuAcα2–6GalNAcα1-O-serine/threonine	G	1:8

[a] *A* Gift from Dr. F. Ramaekers [25, 27–29, 36]; *B* Zymed Laboratories, San Francisco, USA; *C* Molecular Oncology, Gaithesburg, USA; *D* Immunotech, Marseille, France; *E* gift from Dr. O. Børmer [5]; *F* gift from Dr. S. Funderud [32]; *G* Dako, Glostrup, Denmark; *H* Oncogene Science, New York, USA

[b] Microwave treatment of the sections

Results

Seven of the twelve human diffuse gastric carcinomas were successfully xenografted in nude mice (tumorigenicity rate of 58.3%). Short-term primary cultures were achieved in all (100%) the xenografted lines. Five primary cultures stopped growing after a few passages or were overgrown by murine fibroblasts. Two of the seven short-term primary cultures (28.5%) were established as long-term cultures (GP202 and GP220).

GP202 and GP220 cells have been maintained in culture for 45 continued passages (for nearly 2 years 6 months), and when cultured in medium containing 10% FCS they showed population-doubling times of 35 and 22 h, with a saturation density of 2.3×10^6 and 1.3×10^6 cells/ml at passage 37 and 41, respectively (Fig. 1, Table 2). Their plating efficiency amounts to 70%. Cell growth in agarose revealed that GP202 cells have a high cloning efficiency (nearly 30%) and form colonies after 1 week, whereas GP220 have a lower cloning efficiency (2–10%) and visible colonies are not seen until after a period of 2 weeks.

The morphological characteristics of the cells from cases GP202 and GP220 were similar in the original tumours of the two patients, in the nude mice tumours and in the respective cell lines (Figs. 2, 3). GP202 cells are larger than GP220 cells, show less abundant intercellular junctions at the ultrastructural level and grow in culture as a compact thin monolayer covering the whole culture flask (Figs. 2, 3). The GP220 cells grow preferentially in small clusters attached to the monolayer, with a subpopulation of floating cells (Figs. 2, 3). Both lines have cells containing small mucin vacuoles in the cytoplasm and occasional cells with a typical signet-ring shape (Figs. 2, 3). Both cell lines also had an epithelial cell-type morphology with hexagonal cells in culture, as shown by phase-contrast microscopy (Fig. 2).

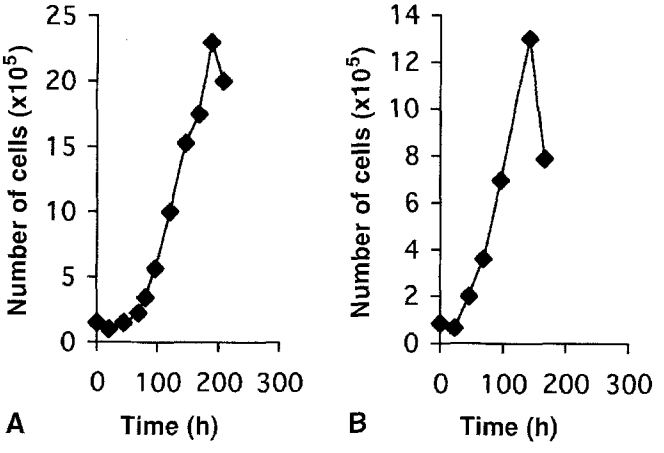

Fig. 1A, B Growth curve of GP202 and GP220 cells in vitro. **A** Growth curve of GP202 cells at passage 40. **B** Growth curve of GP220 at passage 42. Each data *point* represents the mean of triplicated sample cells

Tumour nodules developed at the site of inoculation following subcutaneous injection of 1.2×10^5 GP202 cells from passage 22 in vitro into the superscapular region of N:NIH(s)II-*nu/nu* nude mice. Inocula that became established as growing tumours showed a latency period of 22 days (Fig. 4). During the latency period tumours were neither visible nor palpable. A doubling time of 5 days was observed (Table 2). The GP202 cell line consistently retained signet-ring shaped cells (Fig. 2). The tumours that arose at the site of inoculation were excised before they became large enough for ulceration, haemorrhage or other complications to lead to the death of the host; using this procedure no metastases were observed in any of the nude mice harbouring tumours from the GP202 cell line.

In contrast to the GP202 cell line, the GP220 cell line, after being established as a cell line in vitro, was steadfastly non-tumorigenic in nude mice, despite large inocula of tumour cells (10^7–10^8).

The original tumour for the GP202 cells showed a single aneuploid peak, with a DNA index of 1.785. The respective xenograft exhibited two aneuploid peaks, with DNA indexes of 1.772 and 2.061. The cell line showed only one aneuploid peak, with a DNA index of 2.036. The original tumour from which the GP220 cell line was established had a broad diploid peak, with a DNA index of 1.00, similar to those of the respective xenografted tumour and cell line.

A summary of the immunohistochemical findings is displayed in Table 3. A panel of anti-cytokeratin MAbs was used and confirmed the epithelial origin of the GP202 and GP220 cell lines. Both lines were positive for CK7, CK8, CK18 and CK19. However, the percentage of positive cells for cytokeratins CK8 and CK19 was higher in the GP220 than in the GP202 cell line (Table 3). Both cell lines showed high levels of expression of cathepsin D and type IV collagenase; both were strongly positive

Table 2 Growth characteristics and DNA content of the two cell lines

Cell line	Growth type	Doubling time		DNA content	Tumorigenicity			Delay time (days)[a]
		In vitro (h)	In vivo (d)		Soft agar	nu/nu Tumour	nu/nu Metastasis	
GP202	Adherent	35	5	Aneuploid	+	+	–	22
GP220	Adherent/ floating	22	–	Diploid	+	–	–	–

[a] Number of days to reach a visible and palpable volume of approximately 9 mm^3

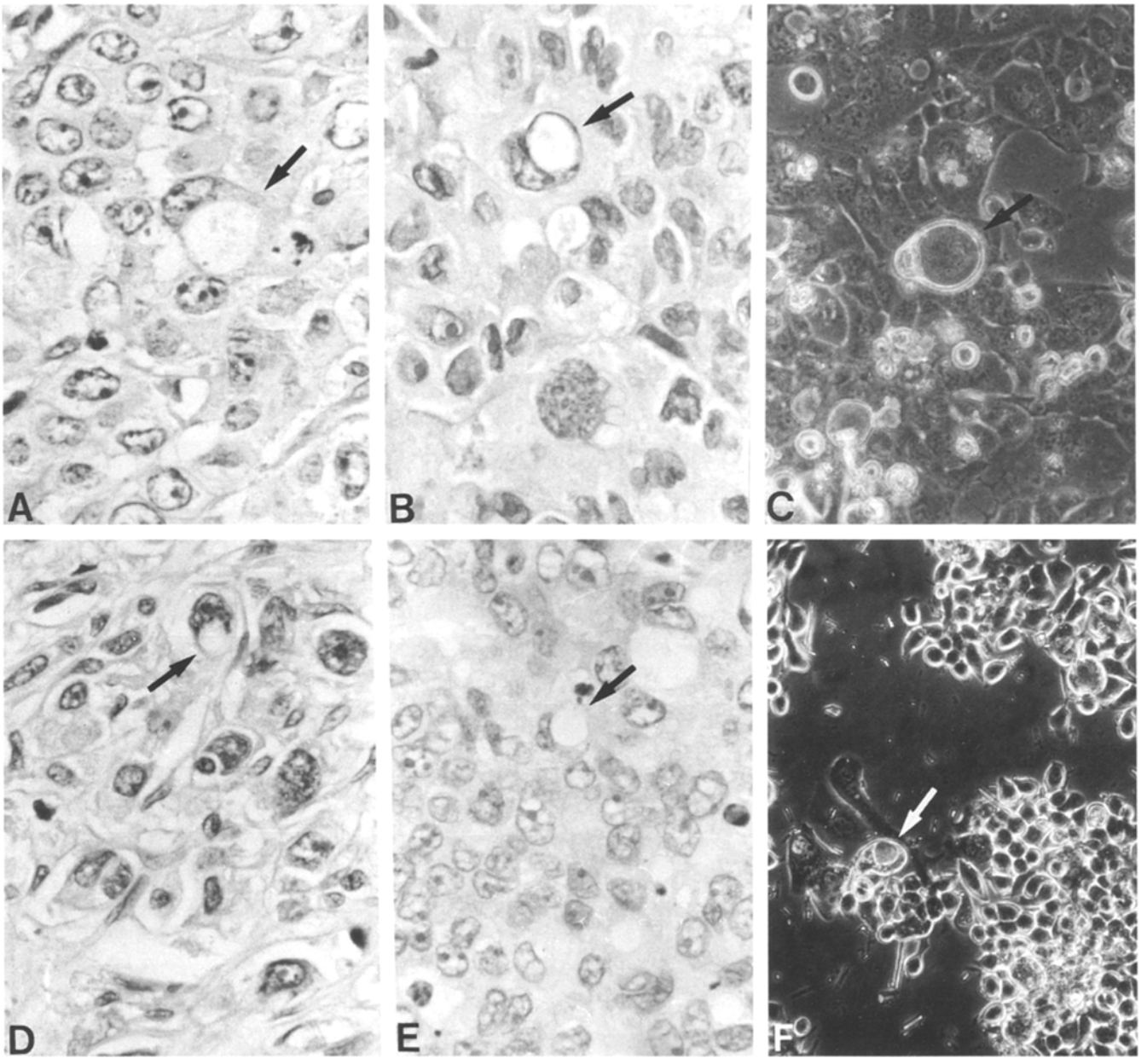

Fig. 2 Source patients' tumours (**A, D**), their respective xenografts in the nude mice (**B, E**), and cell lines GP202 (**C**) and GP220 (**F**). Cases GP202 and GP220 show signet-ring cells (*arrows*) in primary tumours (**A, D**), xenografts (**B, E**) and cell lines (**C, F**). **A, B, D, E** HE, ×700. **C, F** Contrast-phase photographs, ×700

for MUC1 mucin; and both expressed high levels of CEA. The CDw75 antigen and Tn antigen were expressed in more cells from the GP202 than the GP220 line. T antigen was only expressed in the GP202 cell line. Sialosyl-Tn was not expressed in either of the cell lines. Only GP220 expressed EGFR (Table 3).

The comparison between the primary tumours and their respective cell lines showed that both cell lines expressed higher levels of CK7 and lower levels of CK8, CK18 and CK19 than the primary tumours. Loss of expression of EGFR and increased expression of CEA were observed in the GP202 cell line. The expression of CDw75 and T antigens was increased in the GP202 cell line and decreased in the GP220 cell line. Sialosyl-Tn was not expressed in either of the two cell lines, although high levels of expression were detected in the corresponding primary tumours.

We analysed 10 metaphases from xenografts of GP202, and 5 metaphases from xenografts of GP220. GP202 has 72 chromosomes with several numerical and structural abnormalities. GP220 has 46 chromosomes also with several numerical and structural abnormalities (Fig. 5). The karyotypes of GP202 and GP220 cell lines were identical to those observed in the respective xenografts.

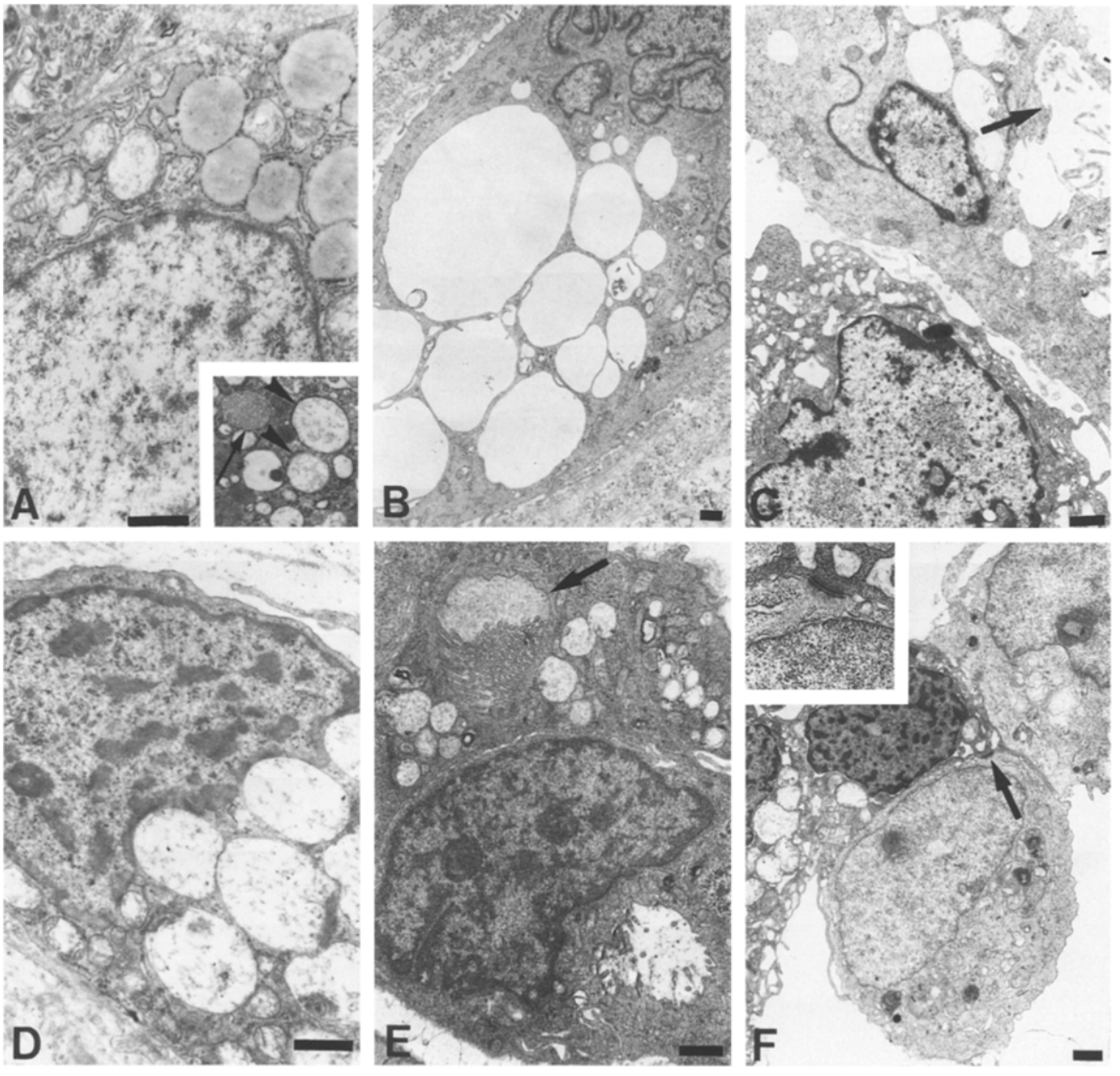

Fig. 3 Source patients' tumours (**A, D**), their respective xenografts in the nude mice (**B, E**), and cell lines GP202 (**C**) and GP220 (**F**). Tumour cells show cytoplasm with abundant rough reticulum (**A**) and mucin granules (**A, D, E**). **A** *Insert* granules with punctate-cerebroid (*arrow*) or floccular structure (*arrowheads*). **C, E** Intracellular lumina (*arrow*). **F** Intercellular junctions of the desmosome type in GP220 cell line (*arrow* and higher magnification in *insert*)

Fig. 4 Growth of GP202 cells in nude mice. Mice were inoculated subcutaneously with 1.2×10^5 GP202 cells on day 0 and the tumour volume determined at the indicated times. Points represent the mean values of 5 mice

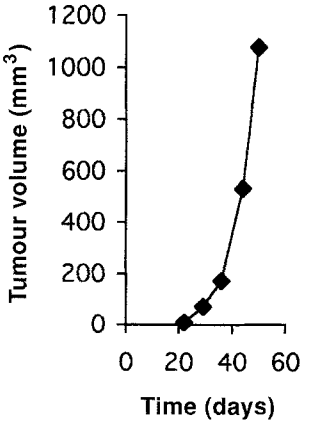

Table 3 Immunohistochemical profile of the primary tumours and corresponding cell lines (– negative, +/– ≤5% positive cells, + >5%–25%≤positive cells, ++ >25%–50%≤positive cells, +++ >50% positive cells)

Antigen	GP202		GP220	
	Primary tumour	Cell line	Primary tumour	Cell line
CK7	+	++	+	++
CK8	+++	±	+++	+
CK10	–	–	–	–
CK13	–	–	–	–
CK18	+++	++	+++	++
CK19	+++	±	+++	++
Cathepsin D	+++	+++	+++	+++
Collagenase IV	+++	+++	++	+++
MUC1	+++	+++	+++	+++
CEA	+	+++	+++	+++
CDw75	–	++	+++	+
Tn	++	++	+	+
T	+	++	+	–
EGFR	+	–	+	+
Sialosyl-Tn	+++	–	++	–

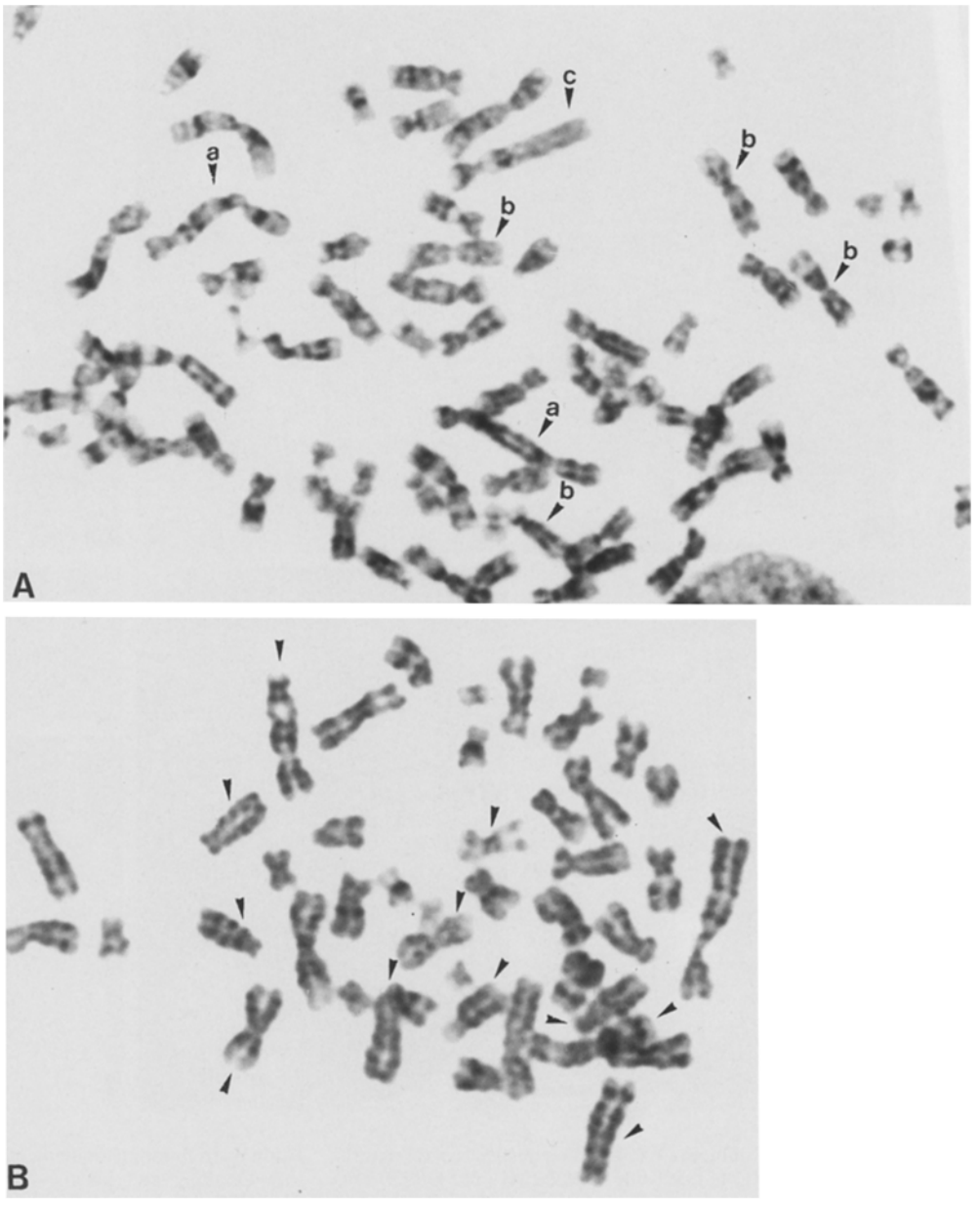

Fig. 5 **A** Metaphase spread from the GP202 cells showing telomeric associations between different chromosomes (*a*); structural abnormalities involving different chromosomes (*b*) and HSR located at chromosome arm 11q (*c*). **B** Metaphase spread from the GP220 cells, showing structural abnormalities of different chromosomes

Discussion

In the present work we have established two human diffuse gastric carcinoma cell lines from xenografted tumours serially passaged in nude mice. Of the 12 diffuse carcinomas xenografted in nude mice, 7 were tumorigenic and only 2 were established as long-term in vitro cultures.

The percentage of successful xenografts we obtained (58.3%) is higher than the previously reported 30.9% success in poorly differentiated carcinomas [34]. Takao et al. obtained a higher success rate with metastatic tumours (47%) than with primary tumours (41%), but the relative frequency of poorly differentiated carcinomas in the two groups is not specified [34].

The similarity between the cell lines and the respective primary tumours regarding the morphology of the cells, at both optic and ultrastructural levels, and the pattern of immunohistochemical expression of cytokeratins and other antigenic markers suggests that the cell lines we have established have retained main of the fundamental biological characteristics of the source patients' tumours. In keeping with this assumption, one of the cell lines (GP220) also maintained the same diploid pattern as the original primary tumour. The other cell line (GP202) displays an aneuploid cell population that was present in the xenografted tumour but was not detected in the patient's primary tumour. In this case it is impossible to know whether the cell line population was present in the primary tumour at undetectable levels or whether it originated de novo at the heterotransplantation phase of the tumour in the nude mice. The similarity of the morphology and antigenic profile of the cell line and the

primary tumour is a point in favour of the former hypothesis.

The GP220 cell line is the first diploid gastric cancer cell line derived from a human diffuse gastric carcinoma on record, to the best of our knowledge. Earlier studies conducted by our group have demonstrated that many diffuse gastric carcinomas are diploid or near-diploid [9], but all the cell lines established from this type of tumour to date are aneuploid [12, 33, 40]. Most of the diffuse-type gastric carcinoma cell lines reported in the literature were derived from cancer cells collected in lymph node metastases [34] or ascitic fluid [12, 17, 33, 37]; these procedures may have introduced a bias in the selection of particular tumour cell subpopulations.

Only four of the cell lines on record were established directly from primary tumours of the diffuse type [19, 26, 38, 40], and the cell lines described here are the first obtained from primary tumours xenografted in nude mice. The morphological and antigenic characteristics of the cell lines we have established are very similar to those of in vivo tumours, suggesting that the model of heterotransplantation of primary tumours in the nude mice may help to increase the pool of diffuse-type gastric carcinoma cell lines.

The profile of cytokeratins expressed in the two cell lines is typical for cells derived from simple non-squamous epithelia [14, 21]. Cytokeratins 8 and 18, the first cytokeratins expressed during early fetal development [18], are routinely expressed in adenocarcinomas of the stomach, pancreas and lower oesophagus, and also in the normal nonstratified epithelia of the digestive tract and its associated glands and ducts [17, 21, 25]. Cytokeratin 19 is also found in a wide range of simple epithelia [15]. The two cell lines, GP202 and GP220, showed lesser expression of CK8, CK18 and CK19 than the respective primary tumours. In contrast, increased expression of CK7 was observed in the two cell lines. Osborn et al. [25] reported the presence of CK7 in gastric adenocarcinomas lacking the expression of intestinal-type markers, thus contradicting their own previous report on the absence of CK7 expression in gastric cancer cases [24]. The data we have obtained, both in the present study and in the study of gastric carcinomas in vivo (unpublished results), do not support the assumption that the absence of CK7 is typical of gastric carcinomas [24] and gastric cancer cell lines [17]. Additional data should be collected in order to see whether or not the expression of CK7 is associated with any particular type(s) of cellular differentiation of gastric cancer cells.

The two cell lines we have established display high levels of expression of CEA, which is in accordance with previous reports on human gastric tumours and gastric carcinoma cell lines [4, 11, 20, 22, 35]. The high levels of expression of cathepsin D and type IV collagenase in the GP202 and GP220 cell lines also fits in well with in vivo findings on gastric carcinomas [8, 30]. It remains to be clarified whether the expression of cathepsin D is specifically associated with the diffuse type of gastric carcinomas, as suggested by Saku et al. [30].

The comparison between the GP202, tumorigenic, cell line and the GP220, non-tumorigenic, cell line disclosed the presence of higher levels of expression of the antigens CDw75, Tn and T (Thomsen-Friedenreich) in the former than in the latter. Both CDw75 and T antigens are associated with an aggressive behaviour of gastric carcinomas [6, 7] and might well be implicated in the tumorigenicity of the GP202 cell line. It remains to be determined whether or not the lack of EGFR in the GP202 cell line is related to its tumorigenicity (as it might be if the growth of the cells of this line, in contrast to those of GP220, is independent of the usual growth stimuli).

In conclusion, we think the establishment of the two cell lines described in this paper represents a valuable addition to the small number of diffuse gastric carcinoma cell lines currently available. Furthermore, the existence of clear-cut differences in several important features between the two cell lines offers an excellent model for studying some of the mechanisms involved in the carcinogenic process of the diffuse type of human gastric tumours.

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